

POTENTIAL ZOO NOTIC RISK OF *GIARDIA DUODENALIS* INFECTIONS FROM
LIVESTOCK AND COMPANION ANIMALS

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Transmission of *Giardia duodenalis* infections from animals to humans has been a subject of recent research interest. Several reports have shown animals harboring strains of *G. duodenalis* that have also been identified from human samples. Much of the work assessing the organism's zoonotic potential has been done on livestock and companion animals, given high prevalence rates, high intensity of shedding, and their relationships with humans as domesticated species. The research presented in this dissertation is a compilation of four complementary studies aimed at assessing the zoonotic potential of *Giardia duodenalis* infections in dairy cattle and dogs.

The first study was a longitudinal cohort study in which the incidence of *Giardia* infections was evaluated in dairy cattle in the New York City Watershed encompassing Delaware, Greene, Schoharie, Ulster, and Sullivan counties in New York. Three cohorts were analyzed based on their prior infection status with *Giardia*. 10,672 fecal samples were collected from calves and adult cattle from 40 dairy herds. The cumulative incidence was 25% over the course of the two-year study. Risk factors such as age, prior infection status, and season of sampling were shown to be significantly associated with shedding *Giardia* cysts.

The second study was a repeated prevalence study done on the same target population described in the first study. The major goal was to determine the prevalence of assemblages of *G. duodenalis* in dairy cattle in order to genetically characterize infections. 2,109 samples were

collected of which 504 were positive for *Giardia* based on fecal flotation for a cumulative incidence of 23.9%. Polymerase chain reaction (PCR) of the beta-giardin and triosephosphate isomerase genes with subsequent DNA sequencing revealed livestock-specific and potentially zoonotic genotypes in dairy cattle in the New York City Watershed.

The third study was a cross-sectional study with aims to 1) determine the prevalence of *G. duodenalis* in dairy cattle in Trinidad and Tobago, 2) conduct multi-locus characterization of *G. duodenalis* infections using PCR, and 3) identify risk factors associated with *G. duodenalis* infections in this population. One hundred and ninety-six samples were analyzed by PCR of the small-subunit ribosomal RNA (*ssu-rRNA*) gene as the only diagnostic test. Twenty-five samples were positive for a prevalence of 12.8%. Of the *ssu-rRNA* positive samples, ten were positive by PCR amplification of the beta-giardin gene. Three samples showed heterogeneity of assemblage typing between the two loci, likely indicating mixed infections of assemblages A and E.

The final study was a cross-sectional study done on owned, shelter, and free-roaming dogs in Trinidad and Tobago. The major goals were to 1) determine the prevalence of *G. duodenalis* infections in dogs, 2) assess the risk of *G. duodenalis* infections to humans through genetic characterization of isolates, 3) compare test agreement between a commercially-available ELISA test kit for *Giardia* and PCR, and 4) identify risk factors associated with *G. duodenalis* infections in dogs. 104 samples were analyzed by PCR of the *ssu-rRNA* gene of which 26 were positive for a prevalence of 25%. No zoonotic strains were present in the study population. Dog-specific assemblages C and D were found as well as one sample identified as assemblage E. The kappa statistic for agreement between PCR and ELISA was 0.67 indicating good agreement between the tests. Risk factors for infection in dogs included age, group housing, and ownership status.

These findings contribute to greater understanding of the zoonotic potential of *G. duodenalis* infections in dairy cattle and dogs. The New York Watershed studies highlight growing public health concerns about zoonotic pathogens in the public water supply. The work done in Trinidad and Tobago represents pilot epidemiologic research on *G. duodenalis* infections from livestock and companion animals and contributes to our knowledge about the global distribution of *G. duodenalis*. While these studies do not directly link infections in dairy cattle and dogs to humans, they provide information on risk factors that may perpetuate *G. duodenalis* infections in these populations, other animal populations and humans. Taking significant risk factors into consideration may reduce the occurrence of *G. duodenalis* infections in dairy cattle and dogs, thereby indirectly reducing the potential for infections in humans.

BIOGRAPHICAL SKETCH

Miguella Paula-Ann Mark-Carew was born on February 13th in Washington D.C. during the great blizzard of 1983. She grew up in Santa Cruz, Trinidad and Tobago for the first five years of her life. She moved to Long Island, New York with her mother in 1988 and lived there until she started college in 2000. Miguella attended Dartmouth College and graduated with a bachelor's degree in Biochemistry and Molecular Biology in 2004. Her love for research grew from various opportunities across different institutions including Brookhaven National Laboratory, Cornell University College of Veterinary Medicine, the National Institutes of Health, and Weill Cornell Medical College.

In 2007, Miguella was accepted to the field of Comparative Biomedical Sciences at Cornell University and joined the laboratory of Dr. Hussni Mohammed. Her training in epidemiology led her back to Trinidad and Tobago as a U.S. Student Fulbright awardee to investigate *G. duodenalis* infections in dairy cattle and dogs in the country. She plans to continue with public health research to improve the health and well-being of humans and animals alike.

To my parents, Marlene and Prior, to my family and friends, and to science:

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“Gratitude is the memory of the heart.”

Jean Baptiste Massieu

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CHAPTER ONE

INTRODUCTION

Giardia is a cosmopolitan intestinal parasite belonging to the order Diplomonadida and the family Hexamitidae (Thompson and Monis, 2004). It is a unicellular protozoan with characteristics similar to anaerobic prokaryotes in that it lacks common eukaryotic subcellular compartments such as mitochondria, peroxisomes, and a traditional Golgi apparatus (Plutzer et al., 2010). The parasite has a simple life cycle and comes in two stages. The motile, bilaterally symmetrical trophozoite stage is ellipsoidal in shape, measures 12-17 by 7-10 μm , and has a ventral disc for attachment to the lumen of the small intestine (Zajac and Conboy, 2006). It is binucleate with four pairs of flagella and a pair of median bodies. The infective cyst stage is hardy, measures 9-13 by 7-9 μm , and can remain in the environment for many months once excreted in the host's feces (Huang and White, 2006).

The genus *Giardia* consists of six species, five of which have host specificity. *Giardia agilis* infects amphibians, *G. ardeae* and *G. psittaci* infect birds, *G. muris* infects rodents, and *G. microti* infects voles and muskrats. *G. duodenalis* (synonymous with *G. lamblia* and *G. intestinalis* in the literature) has a wide host range of mammals including wildlife, livestock, companion animals, and humans. Within *G. duodenalis* are further genetic groupings called assemblages, two of which are considered potentially zoonotic. Assemblages A and B infect humans and a variety of mammalian species, assemblages C and D infect domestic and wild canids, assemblage E infects hoofed livestock (domestic ruminants and pigs), assemblage F infects cats, assemblage G infects mice and rats, and assemblage H infects seals (Lasek-Nesselquist et al., 2010).

Giardia is the causative agent of giardiasis, one of the most common enteric diseases of humans in developed and developing countries. With a low infective dose of 10 cysts, an estimated 2.8×10^8 *Giardia* infections occur each year (Rendtorff, 1954; Lane and Lloyd, 2002).

It is also a commonly-cited cause of diarrheal disease in livestock and companion animals. Typical symptoms include acute and chronic diarrhea, dehydration, abdominal colic, villus atrophy, and intestinal malabsorption of sodium, glucose, and water, though, asymptomatic infections occur frequently (Farthing et al., 1993; Thompson et al., 1993). Acute giardiasis develops after an incubation period of 1 to 14 days and lasts one to three weeks. The physiology of *Giardia* infections is not well-understood. A major cytopathic effect is increased epithelial permeability due to disruption of peripheral membrane proteins, such as the tight junction-associated protein zonula occludin-1 (Buret et al., 2002). This triggers inflammatory responses leading to brush border injury and disaccharidase deficiencies. Additionally, *Giardia* is known to trigger enterocyte apoptosis (Troeger et al., 2007).

Transmission of *Giardia* infections occurs through the fecal-oral route from ingestion of cyst-contaminated food or water or through direct physical contact (Farthing et al., 1993). Cysts are triggered to excyst in the duodenum by exposure to low pH and pancreatic hydrolytic enzymes resulting in the release of two trophozoites. Trophozoites replicate by binary fission and colonize in the proximal small intestine. By mechanisms not fully understood, they are stimulated to encyst, likely due to high levels of bile, cholesterol starvation and pathophysiological events (Gillin et al., 1987; Lujan et al., 1996; Ortega and Adam, 1997). Eventually, cysts are passed in the feces to continue the life cycle.

Many diagnostic techniques are used to identify *Giardia* in contaminated samples. Traditional approaches involve zinc sulfate flotation with subsequent microscopy to observe cysts and/or trophozoites. The sensitivity and specificity of detection of *Giardia* by light microscopy is directly associated with the skills of qualified diagnosticians. Due to the very small size of the parasite, it may be difficult to identify *Giardia* by eye alone. Enzyme-linked

immunoabsorbent assays (ELISAs) have been shown to be very accurate in diagnosing *Giardia* infections. Additionally, they are easily interpretable, cost-effective, and easy to use. Polymerase chain reaction (PCR) is arguably the most sensitive and specific test available. Furthermore, it allows for genetic analyses of the types of assemblages and subassemblages found in samples. Other diagnostic techniques that can be used include fecal smears and direct immunofluorescent assays. Selection of diagnostic methods is a critical step when determining infection rates among populations. It is, therefore, important to choose the most sensitive and specific test(s) available.

Utilization of molecular diagnostics for identification and characterization of *G. duodenalis* infections can involve gene targeting, comparing results at different loci, and using downstream procedures that allow for appropriate grouping of infections at the assemblage and/or subassemblage level. Four *Giardia* genes have been routinely used for molecular diagnosis of *Giardia* infections: the glutamate dehydrogenase (*gdh*) gene, the triose phosphate isomerase (*tpi*) gene, the beta-giardin (*bg*) gene, and the small-subunit ribosomal RNA (*ssu-rRNA*) gene. Primer sequences and applications for the *bg*, *tpi*, and *ssu-rRNA* genes are shown in Table 1. The *ssu-rRNA* gene is a conserved gene and has been traditionally used for species and assemblage differentiation. The *tpi* and *bg* loci have been used mostly for genotyping and subtyping (Feng and Xiao, 2011).

Giardiasis has a significant impact on global public health because of high prevalence and disease burden of infections. Outbreaks of giardiasis most frequently occur through waterborne transmission from contaminated water sources or at recreational water sites, or from person-to-person transmission at day-care centers (due to contact with young children) (Ayalew et al., 2008; Castro-Hermida et al., 2009; Daly et al., 2009; Almeida et al., 2010). A recent review reported 132 waterborne outbreaks of giardiasis since 1954, 108 of which were related to

Table 1. Target gene, primer sequences, assay type, and main uses of three commonly used genes for genotyping *Giardia* (modified from Fend and Xiao, 2011).

Gene	Primer sequence	Size	Assay Type	Usage(s)
<i>tpi</i>	AL3543 - AATATGCCCTGCTCGTCG	605	Nested PCR, DNA sequencing	Genotyping, subtyping
	AL3546 -CAAACCTTITCCGCAAACC			
	AL3544- CCCTTCATCGGIGGTAAC	532		
	AL3545- GTGCCACCAACICCCGTGCC			
<i>ssu-rRNA</i>	RH4- CATCCGGTCGATCCTGCC	292	(Nested) PCR, DNA sequencing	Genotyping
	RH11- AGTCGAACCTGATTCTCCGCCCCAGG			
	GiarF- GACGCTCTCCCCAAGGAC	130		
	GiarR- CTGCGTCACGCTGCTCG			
<i>bg</i>	G7- AAGCCCGACGACCTCACCCGCAGTGC	753	Nested PCR, DNA sequencing, RFLP	Genotyping, subtyping
	G759- GAGGCCGCCCTGGATCTTCGAGACGAC			
	GiarF- GAACGAACGAGATCGAGGTCCG	511		
	GiarR- CTCGACGAGCTTCGTGT			

contaminated drinking water, 18 related to recreational water, and 10 related to foreign travel (Karanis et al., 2007). Reports of giardiasis from foodborne outbreaks are infrequent and have been attributed to poor sanitary practices of food handlers and consumption of contaminated food (Mintz et al., 1993; Hoffmann et al., 2007).

The role of animals in the spread of giardiasis to humans has been controversial (Hunter and Thompson, 2005). The occurrence of *Giardia* in wildlife, particularly, in aquatic mammals like beavers and voles, has been the most important factor for incriminating the organism as a zoonotic agent (Dixon et al., 1997; Appelbee et al., 2005). This originated from investigations of waterborne outbreaks of giardiasis and reports of the disease in campers and hikers who recalled drinking from streams and rivers. Consumption of raw surface water is a clear risk factor for giardiasis. However, contamination of the water supply can come from a variety of sources including wildlife, livestock, and humans.

A great deal of research has focused on the role of livestock, particularly, dairy cattle, as zoonotic reservoirs for *G. duodenalis* due to the presence of farms in watershed catchments. Early research focused on the parasite's high prevalence and the high output of cysts into the environment from infected animals. With great advancements in molecular biology, namely PCR and DNA sequencing, assemblage- and subassemblage-typing of *G. duodenalis* can be implemented to detect and differentiate zoonotic and non-zoonotic strains. Recent studies have shown that infection rates with assemblage A in dairy cattle may be higher than what were previously believed (Feng and Xiao, 2011). A major longitudinal study done in the United States on dairy cattle from birth to 24 months of age showed that the cumulative infection rate of assemblage E was 100% by seven weeks and that of assemblage A was 70% by 15 months (Santín et al., 2009).

The potential transmission of *G. duodenalis* infections from companion animals to humans has also been studied, mostly in dogs. Though dogs are routinely infected with dog-specific assemblages C and D, modest to high infections rates of assemblage A have been documented in Europe and Asia (Leonhard et al., 2007; Inpankaew et al., 2007; Sprong et al., 2009). Assemblage B is less commonly reported in dogs (Minvielle et al, 2008; Claerebout et al, 2009; Traub et al., 2009). It has been suggested that there are two transmission cycles in dogs: one in which there is only dog-specific transmission among dogs and another in which assemblage A is transmitted from pet dogs to humans. The former idea is favored by intensive contact among large numbers of dogs living together whereby dog-specific assemblages outcompete the transmission of other genotypes. For household dogs, the frequency of dog-to-dog transmission may be lower allowing for assemblage A infections to persist (Thompson and Monis, 2004). This theory was supported by a study in which assemblage A was identified at higher rates in household dogs while assemblages C and D were more prevalent in kennel dogs (Leonhard et al., 2007).

Understanding the potential of *Giardia duodenalis* as a zoonotic pathogen requires consideration of the pathogen, its hosts, and the hosts' environments. A multifaceted approach that encompasses thorough data collection, molecular epidemiological analyses, and statistical analyses will contribute to greater understanding of how the parasite propagates through human and animal populations.

Water for residents of New York City comes from two watersheds located in central and southeastern New York, providing almost 2 billion gallons of water each day to an estimated nine billion people. The Catskill/Delaware Watershed, the larger of the two watersheds, spans five counties and provides the majority of New York City's drinking water. Water from this

catchment is currently unfiltered due to the sheer cost of such an undertaking. Growing concern about public health due to lack of a filtration system resulted in the New York City Watershed Memorandum of Agreement (1997), allowing New York City to sidestep implementation of water filtration through development of a comprehensive watershed plan “that maintains and enhances the quality of the New York City drinking water supply system and the economic vitality and social character of the Watershed communities.” The New York City Watershed provides an ideal setting for understanding the risk of *Giardia* infections from livestock, mainly dairy cattle, given the vast number of dairy farms in the area.

G. duodenalis infections have been identified in many different species all over the world. Yet, there is a lack of information about infections in humans or other mammalian species in the Caribbean. Therefore, little is known about the prevalence of the parasite, the strains of *G. duodenalis* that exist, or risk factors that may be associated with infections in the region. Trinidad and Tobago, the southernmost island in the Caribbean, is one of the largest countries in the chain and has an established agricultural system allowing for investigation of *G. duodenalis* infections in dairy cattle. Additionally, the risk of *G. duodenalis* infections from companion animals can be assessed by sampling from the country’s high free-roaming dog population and from dogs at local shelters on both islands.

This dissertation is a collection of four unique studies designed to build on knowledge of *Giardia* infections in dairy cattle and dogs in order to assess the zoonotic risk to humans:

- A longitudinal cohort study to evaluate the incidence of *Giardia* infections in dairy cattle in the New York City Watershed and identify risk factors associated with the occurrence of the parasite and the intensity of cyst shedding.

- A repeated-prevalence study to evaluate the cumulative incidence of *G. duodenalis* infections, identify risk factors associated with infections, and determine the prevalence of *G. duodenalis* assemblages in dairy cattle in the New York City Watershed.
- A cross-sectional study to determine the prevalence of *G. duodenalis* on private and government dairy farms in Trinidad and Tobago, identify risk factors associated with infections, and detect potentially zoonotic strains of *G. duodenalis* in dairy cattle through multi-locus analyses.
- A cross-sectional study to assess the potential zoonotic risk of *G. duodenalis* to humans in Trinidad and Tobago from owned, free-roaming, and shelter dogs, identify assemblages of *G. duodenalis* found in dogs on both islands, and compare the agreement of two diagnostic tests (PCR and ELISA).

Epidemiologic investigation is important in addressing the public health concern of *G. duodenalis* infections to humans from other mammalian hosts. This research will provide additional knowledge to existing information known about the epidemiology of *G. duodenalis* in dairy cattle and dogs and the potential zoonotic risk to humans.

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CHAPTER TWO *

INCIDENCE OF AND RISKS ASSOCIATED WITH *GIARDIA* INFECTIONS IN HERDS ON DAIRY FARMS IN THE NEW YORK CITY WATERSHED

* In the format of Acta Veterinaria Scandanavica

Abstract

The primary aims of this study were to determine the incidence of *Giardia* infections in dairy herds on farms in the New York City Watershed region and to evaluate risk factors associated with infections. Because co-infections of *Giardia* and *Cryptosporidium* spp. are common in this population, we also evaluated the effect of herd infection status on *Giardia* infections.

Farms were grouped into three cohorts based on their prior infection status with *Giardia* and/or *Cryptosporidium* spp. The sampling plan included collecting fecal samples from all calves below 30 days of age and proportional sampling of calves, young stock, and adults. A total of 10,672 fecal samples were collected and analyzed for the presence of *Giardia* cysts using zinc sulfate flotation. Herds enrolled in the study were sampled seasonally for a study period of two years. The probability of shedding cysts past a certain age and the factors that influenced the likelihood of shedding were evaluated using survival analysis. Linear regression was used to evaluate factors that were associated with the intensity of shedding.

Results

The majority of *Giardia* infections occurred in calves within their first 180 days of age, with the most number of calves shedding *Giardia* cysts between 11 and 20 days of age. The incidence of shedding of *Giardia* cysts ranged from 0.0004 per animal day for cattle in the low risk cohort to 0.0011 per animal day for cattle in the high risk cohort. The likelihood of shedding was influenced by the prior infection status of the herd and the season of collection. Infected animals shed on average 9,658 cysts/gram and the intensity of shedding *Giardia* cysts varied significantly with the age ($p < 0.0001$) and the season of collection ($p = 0.0151$ for Spring).

Giardia infections are common in dairy herds in the New York City watershed, particularly in calves less than 6 months of age. Seasonality may be an important factor in the perpetuation of infections based on changes in management practices corresponding to weather patterns of a particular season. A dairy herd's prior infection status with *Cryptosporidium* influences the likelihood of infection with *Giardia*.

Introduction

Giardia is an intestinal flagellated protozoan parasite and has been cited as the most frequent cause of non-bacterial diarrhea in humans. The parasite is a common source of intestinal infections in the developed and developing world with an estimated 2.8×10^8 cases in humans per year [1, 2]. Ingestion of as few as 10 *Giardia* cysts can cause giardiasis [3], and infections are spread via the fecal-oral route by ingestion of cyst-contaminated food or water [4].

The genus *Giardia* has been subject to various taxonomic changes over the years and at one point included over 50 species [2]. As of recent years, there is agreement that six separate species exist: *G. agilis* found in amphibians, *G. ardeae* and *G. psittaci* found in birds, *G. microti* found in muskrats and voles, *G. muris* found in rodents, and *G. duodenalis* found in humans and a wide range of mammalian species [5]. *G. duodenalis* is subdivided into seven assemblages (A-G) that have distinct host preferences (Assemblages C and D for dogs, Assemblage E for livestock, Assemblages F for cats and Assemblage G for domestic rats) [6]. However, Assemblages A and B infect humans and many different species of wildlife, companion animals, and livestock [7].

Research on *G. duodenalis* in livestock, particularly cattle, has shown that the parasite is very common in this population and tends to infect younger calves leading to high prevalence of infection within herds [8, 9]. Studies have reported between 45%-73% of calves 0-24 weeks of

age having infections [8, 10] as well as infection rates as high as 100% [11]. Calves have been reported to be infected with *G. duodenalis* as early as four days of age and have the highest intensity of cyst excretion (10^5 - 10^6 cysts/gram) between the ages of 4-12 weeks [12]. A study done by our laboratory showed a shedding pattern in dairy calves that increased at 4 days of age and peaked at 14 days [13]. Because dairy cattle can shed high levels of *G. duodenalis* and inhabit watershed areas, there has been much concern about the potential risk of zoonotic *Giardia* infections in human populations.

G. duodenalis is etiological agent for diarrheal disease in cattle by itself but is often linked to another common intestinal parasite, *Cryptosporidium* [14, 15]. *Cryptosporidium* spp. are similar to *Giardia* in terms of clinical signs, host range, zoonotic potential, and modes of transmission. Many studies have demonstrated concurrent *G. duodenalis* and *Cryptosporidium* spp. infections in dairy calves [16, 17] as well as in adult animals [18], yet research on the risk of giardiasis due to co-infection with both parasites has not been fully explored.

In 1993, the Environmental Protection Agency (EPA) concluded that New York City (NYC) met the requirements for filtration avoidance. This meant that filtration was not necessary if the water supply met state and federal raw water standards. The NYC Watershed Agricultural Program, created as part of the filtration avoidance, was established to maintain the quality of the water supply and the economic viability of agricultural operations in the region. The program focuses on the management of pesticides, sediment, nutrients such as nitrogen and phosphorus, and pathogenic organisms such as *Giardia* and *Cryptosporidium* spp. as they relate to agriculture and water quality [19]. Our study was initiated to obtain information about the epidemiology of *Giardia* and *Cryptosporidium* spp. in dairy cattle as a means of understanding the potential risk of waterborne outbreaks from these protozoa in New York City.

Many prevalence studies on *G. duodenalis* infection in cattle can be found in the literature while only a handful of incidence studies exist. The primary objectives of our study were to determine the incidence of *G. duodenalis* infections in dairy cattle populations in the New York City watershed region and to evaluate the dynamics of *Giardia* infections by taking into consideration factors that may play a role in the perpetuation of infections at the farm level. Because *Giardia* and *Cryptosporidium* are prevalent in dairy cattle operations, we wanted to assess the risk of *Giardia* infections based on the dairy herd's prior infection status of having animals with *Giardia* and *Cryptosporidium*. Finally, we investigated factors associated with the likelihood of infection with *Giardia* and the intensity of cyst-shedding.

Materials and Methods

We carried out a longitudinal follow-up study to address the stated objectives. The target population consisted of cattle on dairy farms in the Catskill/Delaware Watershed of New York City. The watershed is located in southeastern New York State and houses approximately 200 dairy operations located within the catchment area. The study population was drawn from herds enrolled in a voluntary program administered by the Watershed Agricultural Council (WAC). Approximately half of the herds in the target population were surveyed for the prevalence of *Giardia* in a cross-sectional study [19]. A total of 40 herds were selected from that study population for enrollment in this study based on their willingness to participate.

To account for the initial infection status of the population, study farms were classified into one of three protozoan risk levels based on prior herd infection status [19]. High-risk farms were defined as those on which both *Giardia* cysts and *Cryptosporidium* oocysts were detected (eight farms). Farms on which only *Giardia* was detected were classified as the intermediate risk

group (twenty-five farms). Farms included in the lowest risk category were those on which neither *Giardia* cysts nor *Cryptosporidium* oocysts were previously detected (seven farms). Animals in these herds were randomly sampled using an age stratified sampling design. Three strata of age groups were created: ≤ 6 months of age (calves), 6 to 24 months of age (young stock), and > 24 months of age (adults) [19]. All farms were visited on a seasonal basis (three times a year) for two years starting in June of 1995 and ending in June 1997. Newborn animals, perceived as high risk for infection, were sampled within a week of birth once the decision was made to keep them in the herds. Calves that were negative for *Giardia* infections at the first sampling were sampled again at subsequent farm visits.

Sample Collection and Analysis

Fecal samples were collected rectally from each animal, immediately placed in uniquely labeled specimen containers and stored on frozen cold packs until transported to the laboratory. Once at the laboratory, samples were stored at 4°C. For each sample, three grams of feces were processed using zinc sulfate (1.18 sg) as the flotation medium 20. *Giardia* cysts were enumerated by trained counters using bright field microscopy at 200 \times magnification. An animal was considered *Giardia*-positive if at least one *Giardia* cyst was detected with the correct morphology (i.e. optical properties, internal structure, size and shape). For samples with less than 100 cysts/gram, the entire cover slip was counted. For samples with high cyst numbers, 20 random fields were counted and the estimated cyst count/gram was determined by multiplying by the number of fields on the cover slip. All identification techniques were done at the Cornell University Animal Health Diagnostic Center (Ithaca, NY).

Data collection

Data on intrinsic factors (age, breed, and sex) related to a specific animal were collected by personal interview of the farm owner and examination of herd records. The date of collection was also recorded to adjust for the risk of shedding by season and to determine animal ages.

Statistical Analysis

In the data analyses, the sampling periods were grouped into three seasons representing months with similar weather patterns: winter (November through March), spring (April through June), and summer (July through October). The probability that an animal would shed *Giardia* cysts past a specific time was computed using the survival analysis approach (Statistix 8.0). Kaplan Meier survival curves were used to present the pattern of shedding in relation to the age of the animals, and significant differences between the three risk groups of animals were evaluated using the Log-rank test [21]. The Cox proportional hazard model was used to evaluate the likelihood of shedding in a short time period given the season and prior status of the herd [22]. Because the sampling units in this study, the cattle, are clustered in herds, it was assumed that this clustering would lead to a correlation in the likelihood of infection within the study population. This correlation between responses occurs because they are dependent on exogenous factors that are associated with these responses (i.e. infection with *Giardia*). Conditioning on an observed set of these factors by controlling for their effect in the analysis and including them as covariates in the logistic regression analysis will sometimes achieve approximate conditional independence. However, more often this correlation in the response arises from both observed and unobserved risk factors. It was assumed that the unobserved risk factors were randomly distributed among farms and the overall significance of this assumption was evaluated by using a mixed-effect logistic regression model [23] using Egret software (Cytel Statistical Software, MA).

Regression analysis, with an appropriate log transformation for the number of cysts shed, was employed to identify factors associated with the intensity of shedding of *Giardia* cysts given the age of the animal, the prior risk group, and season of sampling. The significance of a factor was evaluated by the significance of the respective regression coefficient (p-value < 0.05). The analysis was performed using SAS 9.2 (SAS Statistical Software, Raleigh, NC).

Results

A total of 10,672 fecal samples were collected over the course of the study. For statistical analyses, data from 4,938 unique dairy animals from ages 1 day to 730 days of age were used. We diagnosed 1,236 animals as shedding *Giardia* cysts during the study period. The cumulative incidence of *Giardia*, computed as the proportion of new cases of *Giardia* over the course of the two-year study, was 25% (1,236/4,938). Animals in low risk herds had the lowest incidence rate (0.0004 per animal day = 96 new cases/243,501 days) in comparison to animals from moderate risk (0.0006 per animal day = 603 new cases/975,890 days) or high risk herds (0.0011 per animal day = 537 new cases/501,710 days).

Among all animals that were diagnosed as shedding *Giardia* cysts, 95.8% were calves (1,184/1,236). The earliest age at which a calf shed *Giardia* was 2 days. Figure 1 shows the frequency distribution of the age of young animals up to 6 months of age (≤ 180 days) that were diagnosed as shedding *Giardia* cysts among the study population. The most number of calves began shedding *Giardia* between 11 and 20 days of age (220 animals). The average number of estimated cysts shed was 9,658 cysts/gram with a range from 1 cyst/gram to 1.75×10^6 cysts/gram.

A total of 428 new cases of *Giardia* were diagnosed in the summer, 452 cases in the winter, and 356 cases in the spring. Winter had the highest incidence rate of shedding (0.00083

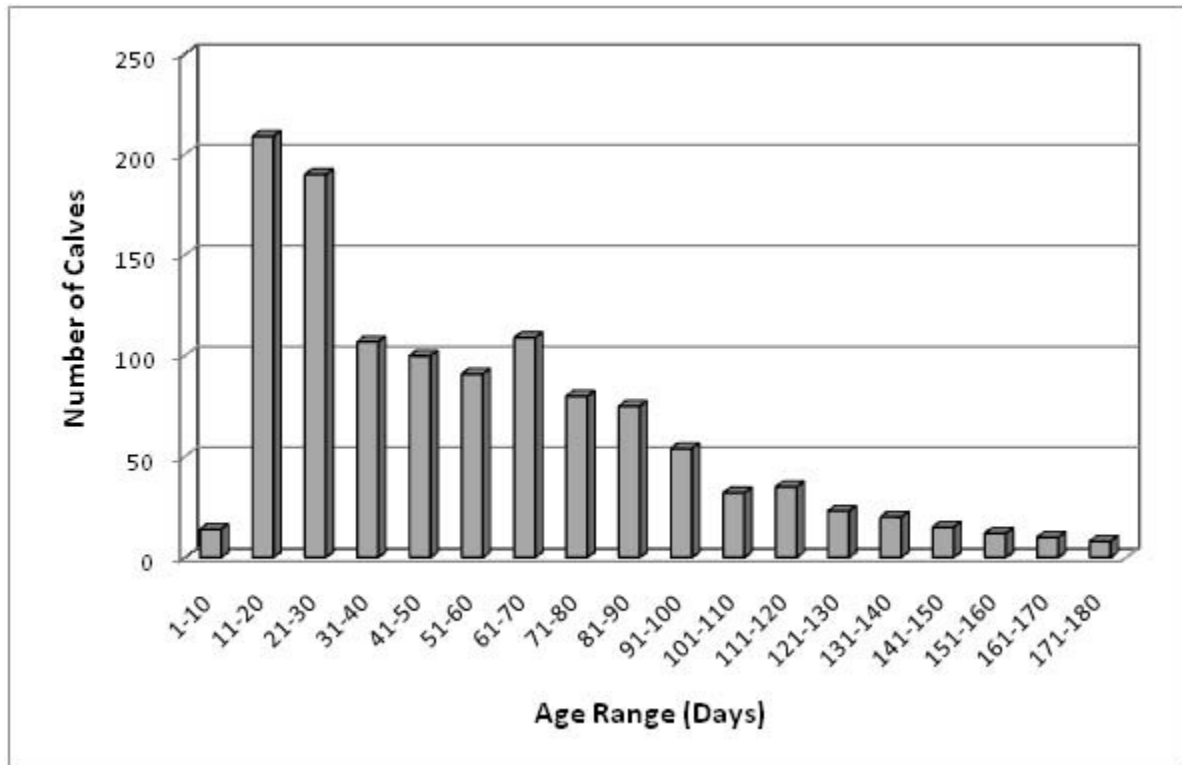


Figure 1. Frequency distribution of the age of animals, up to 180 days of age, among young stock diagnosed as shedding *Giardia* during the study period. The range is measured in ten-day intervals.

per animal day) while spring had the lowest rate (0.00057 per animal day). There were no significant differences between crude incidences among the three seasons. Figure 2 shows the plot of the survivorship function for the likelihood of not shedding *Giardia* cysts over time based on season of sampling. The analysis was limited to animals that were two years of age or less. The probability of shedding *Giardia* past a certain age was higher in winter and summer in comparison to spring ($p < 0.05$) (the survival curve for spring is higher than that for winter or summer). There was no significant difference between winter and summer. We examined the potential likelihood of clustering of shedding of *Giardia* cysts by farm using random effect models. There was no evidence of hierarchical clustering and the likelihood of shedding *Giardia* cysts was randomly distributed by farm (data not shown).

The probability of an animal shedding *Giardia* cysts past a certain age was evaluated among the three risk cohorts using survival analysis (Figure 3). The analysis was again restricted to a follow up period of up to 730 days. All animals had high incidence of shedding *Giardia* cysts earlier in their lives. Ten percent of the animals from farms in the low risk cohort were found to shed cysts within 49 days of age while an equivalent percentage in the moderate and high risk groups become infected within 35 and 27 days, respectively (the 90% percentile of the survival function). There was a significant difference in the risk of shedding among the three groups ($p < 0.05$). The probability of shedding *Giardia* cysts within the first year for the low, moderate, and high risk groups was 21, 31, and 45%, respectively. There was no evidence that there was a clustering of incidence of *Giardia* cyst-shedding by farm beyond chance alone as was evaluated with the mixed effect models analysis (data not shown). The results of investigating the likelihood of shedding *Giardia* cysts in a specific season while controlling for the prior herd risk are shown in Table 1. Animals sampled in spring were less likely to be

Table 1. The impact of season of collection and the prior status of the herd on the hazard of shedding *Giardia* cysts among dairy cattle in the study population

Risk factors	Regression coefficient	Standard error	Hazard ratio	95% CI
Prior risk of <i>Giardia</i>				
<i>Low risk</i>	0		1.0	
<i>Moderate risk</i>	0.328	0.109	1.4	1.1, 1.7
<i>High risk</i>	0.620	0.110	1.9	1.5, 2.3
Season of the year				
<i>Summer</i>	0		1.0	
<i>Winter</i>	0.058	0.080	1.1	0.9, 1.2
<i>Spring</i>	-0.266	0.083	0.7	0.6, 0.9

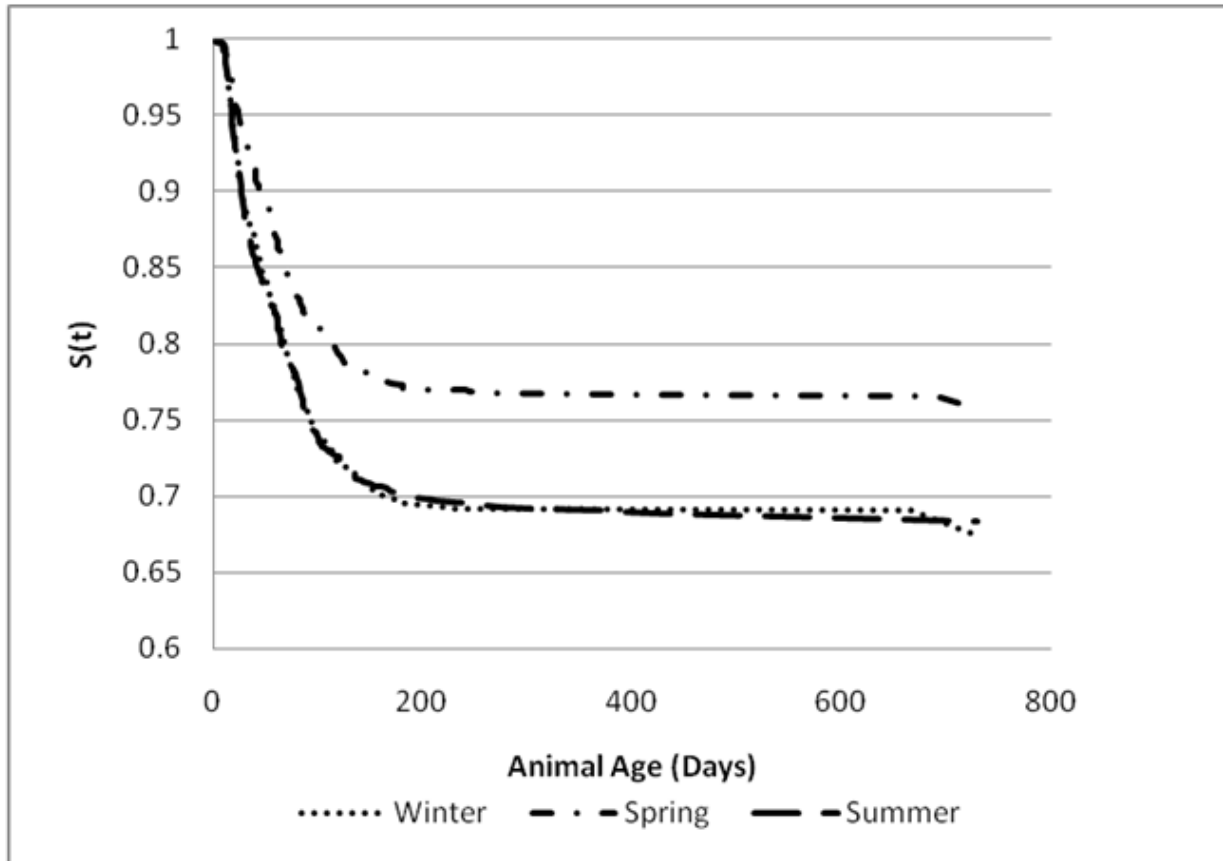


Figure 2. Kaplan-Meier survivorship curve for the probability of not shedding *Giardia* passed certain age for animals enrolled in the study grouped by season of the year.

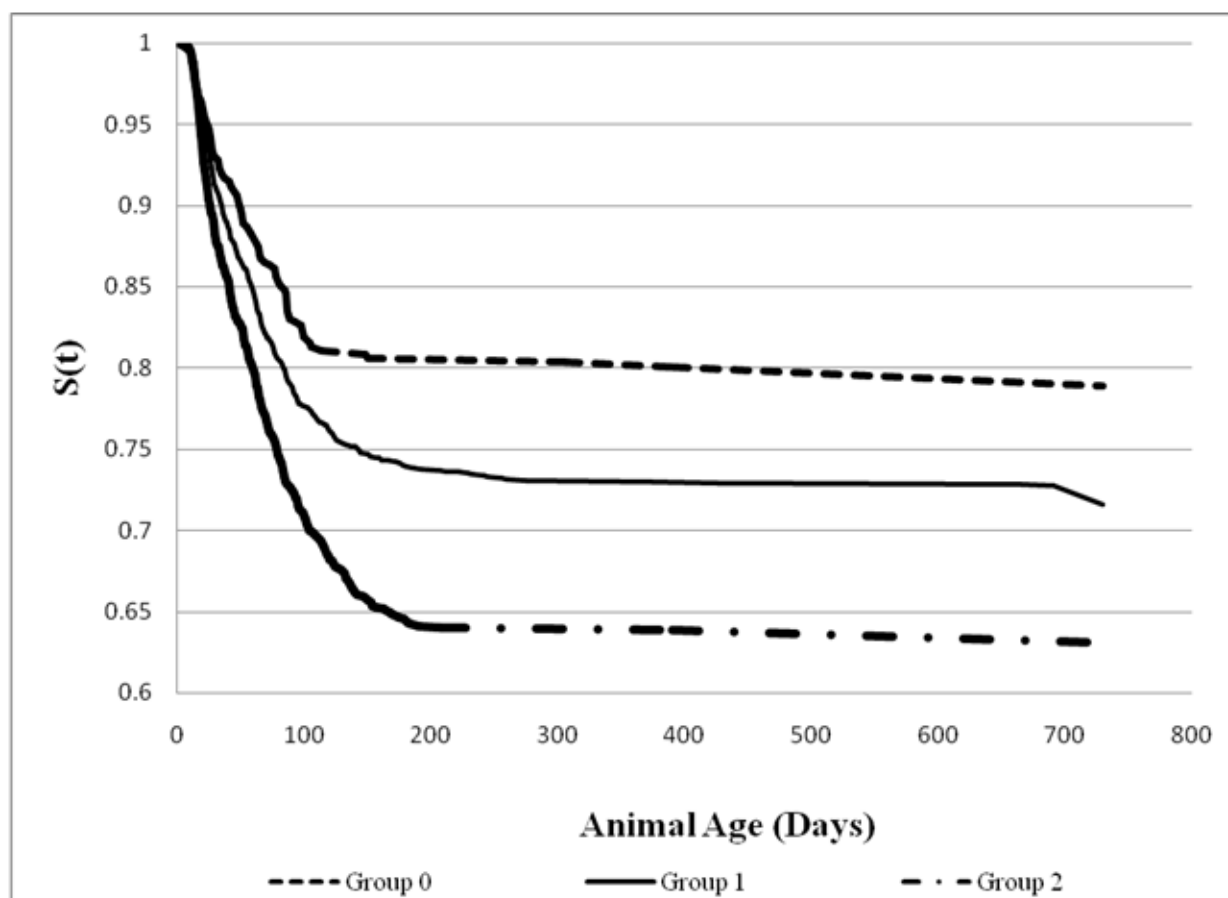


Figure 3. Kaplan-Meier survivorship curve for the probability of not shedding *Giardia* passed certain age for animals enrolled in the study grouped by prior herd infection status.

Risk Factor	Regression Coefficient	Standard Error	Pr > t
<i>Age</i>	-0.0142	0.000980	<0.0001
<i>Age</i> ²	0.000015	1.367E ⁻⁴	<0.0001
Prior Infection Status			
<i>High risk</i>	0		
<i>Low Risk</i>	0.2201	0.0840	0.0089
<i>Intermediate Risk</i>	-0.0288	0.0539	0.5939
Season of Infection			
Summer	0		
<i>Winter</i>	-0.0554	0.0464	0.2324
<i>Spring</i>	-0.1205	0.0495	0.0151
Constant	3.4949	0.0684	<0.0001

Table 2. Factors that affect the intensity of shedding *Giardia* in dairy herds in the New York City Watershed.

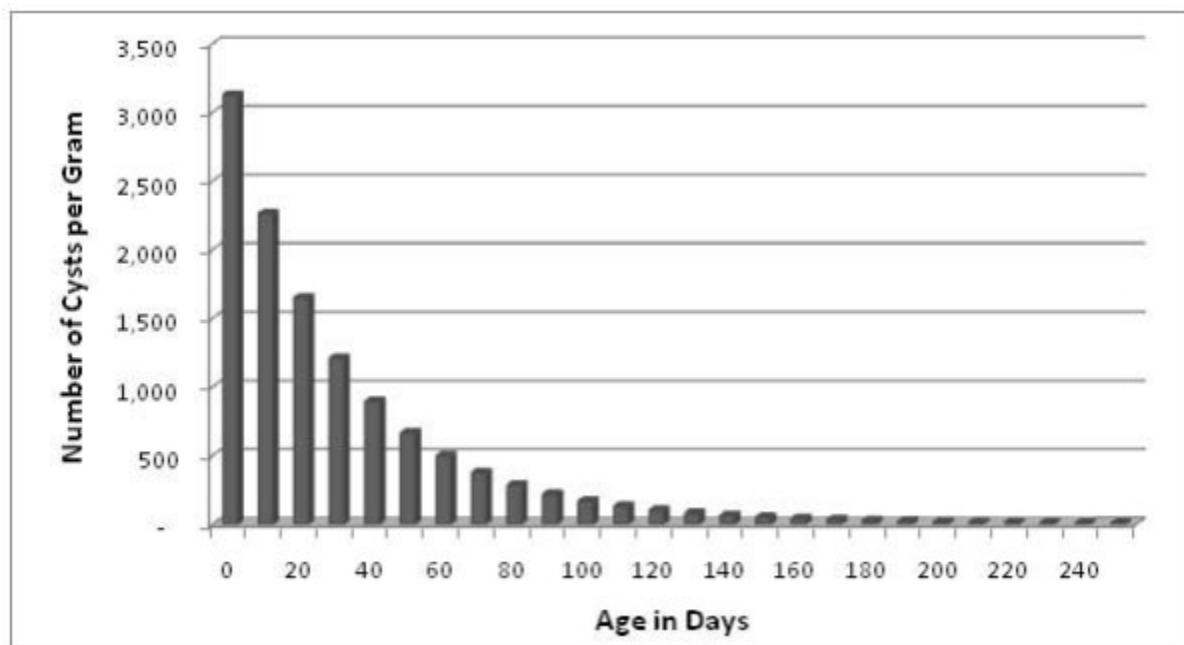


Figure 4. The pattern of intensity of shedding *Giardia* cysts by age of the animal for all animals enrolled in the study and diagnosed as shedding cysts during the follow-up period (730 days).

diagnosed as shedding *Giardia* cysts compared to animals that were sampled in winter (hazard ratio = 0.7). The risk of shedding *Giardia* cysts in animals that were sampled in summer was not significantly different than the risk for animals that were sampled in winter when we adjusted for the herd status prior to enrolling animals. Animals that were sampled from moderate or high risk farms had an increased likelihood of shedding *Giardia* cysts in comparison to animals that were from low risk herds.

Risk factors that were hypothesized to be associated with the intensity of shedding *Giardia* cysts were evaluated (Table 2). The intensity of shedding *Giardia* cysts decreased significantly with the age of the animal (Figure 4). The figure is produced by substituting the age of the animal into the equation: $Y = 3.4949 - 0.0142\text{age} + 0.000015\text{age}^2$ (where Y = log of number of cysts shed/gram of feces). Younger animals were shown to shed higher numbers of cysts and the pattern of shedding decreases with age reaching its minimum value around 250 days. The shedding intensity also varied by the season of the year. Animals sampled in winter and spring shed fewer cysts per gram in comparison to animals sampled in summer (as seen by negative regression coefficients for the former) (Table 2).

Discussion

Several studies have reported on the prevalence of *Giardia* spp. on dairy farms using a cross-sectional study design [12, 24]. Our study differs from these because we used a longitudinal approach in which the occurrence of the protozoa among dairy herds in the watershed was observed over a time period of two years. The approach we undertook, by virtue of its design of repeated sampling, shed more light on the dynamic of the infection in the population compared to a cross-sectional approach which reflects a one-time sampling [22].

Because previous studies have shown associations between *Giardia* and *Cryptosporidium* infections, we assessed the incidence of *Giardia* in dairy herds with a prior history of co-infection with both parasites as compared to herds with a prior history of infections with only *Giardia* and herds without infections with either parasite. Animals in the high risk group had the greatest chance of shedding *Giardia* cysts within their first year in comparison to the low and moderate risk groups. Our analyses showed that the high risk group had the least number of animals that remained *Giardia*-free over the course of two years. It is likely that combined *Giardia* and *Cryptosporidium* infections impacted the probability of shedding and potential for the spread of infection in this group. It is important to note that the number of farms in each risk group was not equal and that the low risk group included the least number of farms. We cannot rule out the possible effect of this on our analyses.

Seasonality was shown to play a significant role in the probability of dairy cattle becoming infected with *Giardia*, particularly during the winter and summer months. Our results are in line with previously published studies that show season being associated with risk of infection in dairy cattle [19, 25]. Seasonal weather patterns influence management practices of dairy farms in the New York City Watershed region in where animals are confined in barns during the winter months and are allowed to roam in pastures during the summer. We speculate that being in close quarters during the winter and having access to streams and grazing land that may be contaminated with *Giardia* cysts from wildlife, other cattle within the herd, and human waste during the summer months may serve to propagate infections during these two seasons. Furthermore, we believe that the probability of infections as well as incidence rates were lowest in the spring because the calving season in this area begins in late summer and continues through October, meaning that young animals were less prevalent in herds.

Results are in agreement with previous studies showing that calves start shedding *Giardia* cysts shortly after birth [24]. Calves began shedding *Giardia* by 2 days of age, three days earlier than we had previously reported [26]. Only 4% of dairy cattle that shed *Giardia* over the course of the study were over 6 months of age.

Age, coming from a herd with a prior history of shedding *Giardia* cysts, and shedding *Giardia* in spring were determined to be risk factors associated with the intensity of shedding *Giardia* cysts. Evidence in support of age being a risk factor for infection is common throughout the literature [19, 27, 28]. Based on microscopic analysis, cysts counts from calves shedding *Giardia* varied from one cyst/gram to as high as 1,759,824 cysts/gram. The animal that shed over a million cysts was 28 days old. The mean number of cysts/gram shed by all cows in the study (including young stock and adults) was 9,658 cysts/gram and by calves only was 10,069 cysts/gram. Our estimates are much higher than other published reports [10, 13, 29] and may be attributed to high prevalence of *Giardia duodenalis* in the New York City Watershed ecosystem.

Calves begin shedding both *Giardia* and *Cryptosporidium* early in their development and can spread infections to other animals within the herd. Additionally, depending on the strain of *G. duodenalis* being shed, there is potential for infections to spread to other animal populations, including humans. The spread of the infection within the farm would contribute to an increase in the herd level of endemicity. Our analyses support this since herds in the moderate and high risk cohorts were more likely to have infections than herds without prior history of infection as shown in Table 1.

This study provides information regarding the incidence of *Giardia* in cattle in watersheds and the dynamics of the infection as they relate to the endemicity of the protozoan in these populations. We believe that *Giardia* and *Cryptosporidium* are epidemiologically linked,

based on their common hosts, mode of transmission and similar clinical manifestations. Though levels of endemicity differ for the two intestinal parasites (*Giardia* infections often being cited as more prevalent in dairy cattle than *Cryptosporidium* infections) [9,17,30], we argue that efforts to control *Giardia* infections in dairy cattle herds in the New York City Watershed should target both protozoa.

Recent studies have shifted from using zinc sulfate flotation to more advanced diagnostic tests such as immunofluorescence antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) [29, 31]. We feel confident that our flotation technique was sufficient for identifying and enumerating *Giardia* cysts, though others have reported that the sensitivity of the test is substantially lower than other techniques [31]. We cannot rule out the possibility that our incidence values are underestimated.

Given that this study was a follow-up to a previously published study where we sought to identify risk factors associated with *Giardia* infections in the target population of dairy cattle in the New York City Watershed, we did not include herd management factors in our analysis [19]. We believe that we accomplished our goals without including these factors but recognize that their addition in our analyses would have strengthened our results.

Future directions point to molecular analyses of fecal samples collected in this study in order to identify assemblages of *G. duodenalis* and to assess the potential for zoonotic transmission of *G. duodenalis*. We understand that the age of the samples may be a hindrance to obtaining this information, but some studies have shown successful PCR amplification of samples as old as ours [32]. Published reports have found Assemblages A, B, and E in dairy cattle worldwide [8, 23, 33]. We plan to contribute novel molecular information to the literature on the types of *G. duodenalis* infections found in dairy herds in the New York City Watershed

through PCR analysis of the triosephosphate isomerase (*tpi*) and beta-giardin (*bg*) genes and subsequent sequencing to identify both zoonotic and livestock-specific strains in this population.

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CHAPTER THREE *

PREVALENCE OF *GIARDIA DUODENALIS* ASSEMBLAGES AMONG DAIRY HERDS IN THE NEW YORK CITY WATERSHED

* In the format of Veterinary Parasitology

Abstract

A longitudinal herd-level study was carried out to determine the cumulative incidence of *Giardia duodenalis* infections in dairy cattle in the New York City Watershed. We also sought to assess the changes in infection pattern of animals diagnosed as shedding *Giardia* over time, determine risk factors that may be associated with *G. duodenalis* infections, and identify potentially zoonotic infections. A total of 2,109 fecal samples were randomly collected from dairy cattle at 34 farms in the New York City Watershed on a seasonal basis. A total of 504 *Giardia*-positive samples were identified by zinc sulfate flotation. The overall cumulative incidence of *G. duodenalis* based on flotation results was 23.9% with 73.8% of all infections occurring in animals under 180 days of age (372/504). The intensity of infection ranged from 2 to 563,200 cysts/gram of feces. Cattle shedding *Cryptosporidium* spp. oocysts were twice as likely to shed *G. duodenalis* cysts in comparison to the animals that did not shed oocysts (1.81 95% CI 1.26–2.60 $p = 0.0012$). In the multivariate analysis, only the age of the animal and the presence of dogs on the farm were significantly associated with the likelihood of shedding *G. duodenalis*. DNA was extracted from positive samples and analyzed by polymerase chain reaction (PCR) of the beta-giardin and triosephosphate isomerase genes of *Giardia* spp. 304 samples were analyzed by PCR of which 131 were sequenced. 22.1% of sequenced samples were identified as assemblage A and 77.9% were identified as assemblage E. Interestingly, 100% of specimens identified as assemblage A were from calves under 84 days of age indicating that younger cattle are important reservoirs for potentially zoonotic assemblages of *G. duodenalis*.

Introduction

Giardia duodenalis is a protozoan parasite of important public health significance in both the developed and developing world. It is the most common intestinal protozoan, causing giardiasis in 200 million people worldwide each year (WHO, 1996). *Giardia duodenalis* has a wide host range parasitizing a variety of mammalian species (Thompson, 2000). Because of its wide host range, the zoonotic potential of *G. duodenalis* should not be neglected. Ingestion of contaminated food and water, particularly from rivers and streams, is of particular concern to public health since as few as 10 cysts may cause infection (Rendtorff, 1954).

Current molecular characterization of *G. duodenalis* assemblages has led to subdivision of the species into eight distinct assemblages (A–H). Humans are infected with assemblages A and B which can also infect wildlife, companion animals, and livestock (Cacciò et al., 2005). Assemblages C and D are specific to dogs, assemblage E to hoofed livestock (such as goats, sheep, pigs, and cattle), assemblage F to cats, assemblage G to rodents (Monis et al., 2003), and assemblage H in marine mammals (Lasek-Nesselquist et al., 2010).

G. duodenalis has been found in cattle all over the world at prevalences as high as 100% from some sampled herds (Xiao and Herd, 1994, Olson et al., 1997, Gow and Waldner, 2006 and Hamnes et al., 2006). Infections with *G. duodenalis* in calves tend to occur towards the end of the neonatal period, though our laboratory has reported calves as young as two days of age shedding *Giardia* cysts (Mark-Carew et al., 2010). Infected calves have been shown to shed high levels of cysts at intensities of 10^5 – 10^6 cysts per gram between 4 and 12 weeks of age (O’Handley et al., 1999 and Ralston et al., 2003). Additionally, calves can excrete cysts for at least 100 days without a significant decline in excretion intensity (O’Handley et al., 1999).

Shedding of assemblages A, B, and E have been reported in dairy cattle. In a study done in New Zealand, assemblages A and B were present in the study population, however, assemblage E, the livestock-specific genotype, was not found (Winkworth et al., 2008a and Winkworth et al., 2008b). Feng et al. (2008) reported 83% of *Giardia* positive specimens from 58 dairy cattle having the zoonotic assemblage A genotype while the remaining 17% had the livestock-specific assemblage E genotype. Trout et al., 2004, Trout et al., 2005, Trout et al., 2006 and Trout et al., 2007 published a series of papers where they assessed the presence of *Giardia* assemblages in dairy cattle of different age groups on farms in seven states in the United States. They reported 15% of pre-weaned calves, 7% of post-weaned calves, 3% of heifers, and 2% of adult cows shedding assemblage A cysts while assemblage E was found in 45% of post-weaned calves, 33% of heifers, and 25% of adult cows. These studies illustrate the heterogeneity of the distribution *Giardia duodenalis* infections among dairy cattle of various ages and show that characterization of *Giardia* species is important in understanding the ecology of the organism and its zoonotic potential.

Recent studies have sought to identify external factors associated with *Giardia* infections in dairy cattle. Age, specific management practices, and environmental conditions have been shown to be significantly associated with infections (O’Handley et al., 1999, Wade et al., 2000, Gow and Waldner, 2006, Winkworth et al., 2008a, Winkworth et al., 2008b and Mark-Carew et al., 2010). In the present study, we set out to determine the prevalence of *Giardia duodenalis* in the New York State Watershed in dairy cattle, assess the zoonotic potential of *G. duodenalis* found in our study population, and to identify risk factors associated with infections.

2. Materials and Methods

2.1. Study design

In our longitudinal epidemiologic study, the target population was dairy herds in the New York City Watershed in Delaware County, New York. A total of 34 farms were recruited and agreed to participate in the study from September 2006 to May 2008. A stratified sampling design was developed to capture the potential variability in the likelihood of shedding *G. duodenalis* in a herd. A maximum number of 20 samples were randomly collected from each farm, 11 to be collected from calves (animals under 180 days of age) and 9 from adult cattle. Samples were taken from all available animals if less calves and adult cattle were on the farms. Any animal that was diagnosed as not shedding *Giardia* was resampled to determine its current infection status (if the animal was still present on the farm during subsequent sampling). The sampling scheme was stratified by season based on similar weather patterns to capture the potential variability in shedding due to the time of the year: Winter (November through March), Spring (April through June), and Summer (July through October).

2.2. Sample collection and analyses

Fecal samples were collected per rectum, put in collection tubes labeled with the animal's identification number and birth date (if available), and stored in frozen cold packs until transported to the laboratory where they were stored at 4 °C until processing. The fecal consistency was identified as one of the following: loose/normal, dry, runny, liquid, or bloody. All samples were processed using a standard quantitative zinc sulfate (specific gravity of 1.18) and sucrose (specific gravity of 1.33) centrifugation concentration flotation to recover *Giardia* cysts and *Cryptosporidium* oocysts, respectively. Ten grams of feces was weighed and dissolved in 150 mL of water. The solutions were sieved with tea strainers then centrifuged (first in water then zinc sulfate or sucrose) in 15 mL tubes to approximately enumerate the number of (oo)cysts/gram feces. Microscopic examination was carried out using bright field and phase

contrast microscopy. An animal was considered positive for infection based on the presence of cysts that were the correct morphology and size as *Giardia*. After microscopic examination, slides were placed in 50 mL tubes filled with water and were stored at 4 °C. To prepare specimens for DNA extraction, slides and cover slips were removed from the tubes, and the tubes were centrifuged at $3000 \times g$ for 15 min at 4 °C. The supernatant was poured off until 5 mL was left in each tube. The pellet was resuspended in water and transferred to 2 mL tubes for storage at -20 °C.

2.3. Data collection

Data on putative risk factors hypothesized to be associated with the likelihood of shedding *G. duodenalis* cysts were collected using a questionnaire. The questionnaires were completed by personal interview of each dairy farm owner/manager and included data on demographic (age, sex, breed of animal), geographic (location of the farm), and management practices (housing, bedding and presence of other animal species on the farm).

2.4. DNA extraction

DNA was extracted from the samples using the DNeasy Tissue Kit (Qiagen, Valencia, CA) with a modified protocol (Santín et al., 2009). 180 µL of ATL Buffer and 20 µL of Proteinase K were added to 1.5 mL tubes containing 50 µL of processed feces. Following an overnight incubation at 56 °C, the manufacturer's protocol was followed. The only exception was that 100 µL of nucleic acid was eluted using AE buffer instead of the suggested 200 µL.

2.5. PCR and DNA analysis

Samples that were positive based on flotation were analyzed using PCR. A 455 bp fragment of the *tpi* gene and a 384 or 511 bp fragment of the *β-giardin* gene (from two different sets of primers) were amplified using nested PCR protocols previously described (Sulaiman et

al., 2003, Cacciò et al., 2002 and Lalle et al., 2005). PCR products were analyzed on 1% agarose gel with ethidium bromide staining. All positive PCR products were purified using Exonuclease I/Shrimp Alkaline Phosphatase (Exo-SAP-IT™) (USB Corporation, Cleveland, OH) and sequenced in both directions in 18 µL reactions at the Cornell University Life Sciences Core Laboratories Center using the Applied Biosystems Automated 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). Sequence chromatograms of each strand were aligned and examined with MEGA4 software (2008). Nucleotide sequence searches were conducted using BLAST (<http://www.ncbi.nlm.nih.gov/blast>).

2.6. Statistical analyses

The significance of association between each of the putative risk factors and the likelihood of *Giardia* spp. in the feces was evaluated using logistic regression analysis in a univariate model. The magnitude of the association was quantified using the odds ratios and interval estimates of the OR were computed using the 95% confidence interval. Factors that were significantly associated with the likelihood of *G. duodenalis* in the univariate analysis were considered further in a multivariate analysis to evaluate the significance of each factor while simultaneously controlling for the effect of other factors. Data were analyzed using SAS 7.0 for Windows (SAS Statistical Software, Raleigh, NC). The significance level for the factors in all models was assessed at $p < 0.05$.

Because the sampling units in this study, cattle, are clustered in herds, it was assumed that this clustering would lead to a correlation in the likelihood of infection within the study population. It was assumed that the unobserved risk factors were randomly distributed among farms and the overall significance of this assumption was evaluated by using a mixed-effect logistic regression model using Egret software (Cytel Statistical Software, MA).

Linear regression, with log transformation of the number of cysts shed/gram feces was used to identify factors that were significantly associated with the intensity of shedding while controlling for the effect of other factors. Because of the potential clustering or correlation in the amount of cyst shed per gram among infected animals within a farm, we evaluated the potential impact of this cluster using mixed effect models. The random effect variable in the analysis was the farm. The analysis was performed using the SPSS software (PAWS 18 Statistics, Mount View, CA).

3. Results

Giardia cysts were detected in 504 of 2,109 samples based on flotation analysis yielding an overall cumulative incidence of 23.9% among sampled herds. A total of 372 of the 504 *Giardia*-positive samples were from animals under 180 days of age (73.8%). Fig. 1 shows the frequency distribution of animals up to 180 days of age that shed *Giardia* cysts over the course of the study. The majority of infected cattle were below 45 days of age, making these young animals a high risk group. The age among animals shedding *G. duodenalis* cysts ranged from 3 days to 3529 days of age. The age at which the greatest number of sampled animals shed cysts was 32 days (mode). The intensity of cyst shedding ranged from 2 cysts/gram to 563,200 cysts/gram of feces.

The within-farm prevalence ranged from 4.8% to 41.8% (data not shown). Of the cattle sampled identified as shedding *Giardia* by flotation, 47.6% to 100% were less than 180 days of age depending on the farm from which they originated. Animals less than 180 days old represented 100% of the *Giardia*-positive samples on six farms. Similar percentages of positive samples were obtained for all sampling seasons. There was no significant seasonal variation in

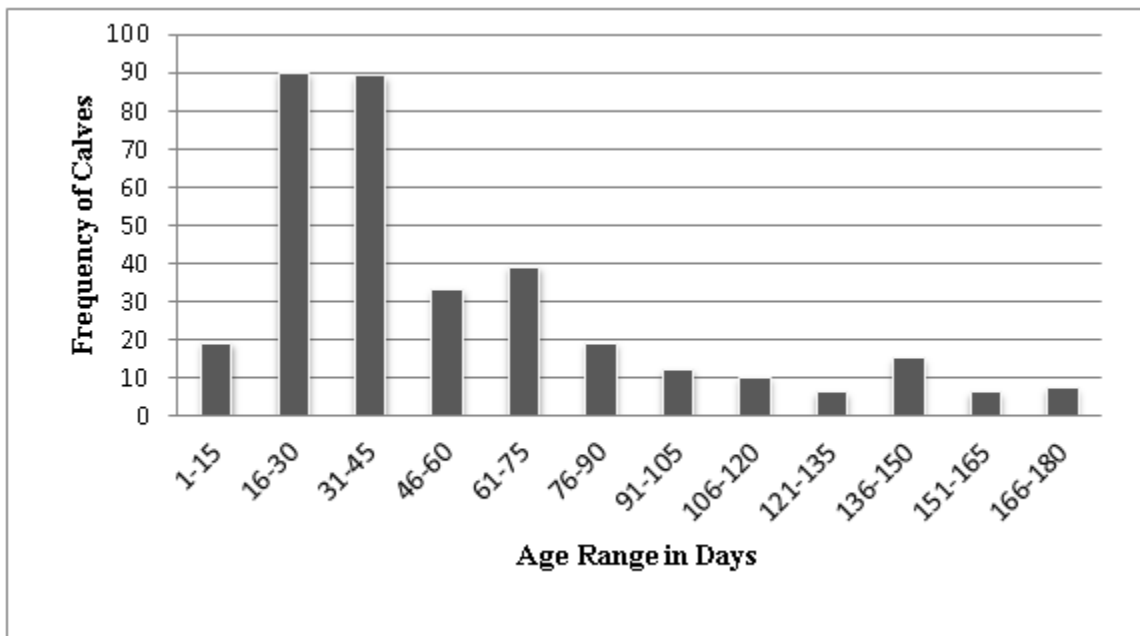


Figure 1. Frequency distribution of number of calves 180 days or less shedding *Giardia duodenalis* infections in the study population.

Table 1. Univariate analysis of risk factors associated with *G. duodenalis* infections in the study population.

Factor	OR	95% CI	p-value
<i>Age</i>			
≤ 31 days	4.6	3.4 – 6.1	< 0.001
31 - 60 days	12.3	8.8 – 17.1	< 0.001
61 – 180 day	4.3	3.3 - 5.8	
> 180 days	1.0		
Co-infection with <i>Cryptosporidium</i> spp.			
Yes	1.8	1.3 -2.6	0.001
No	1.0		
Dogs on Farm			
Yes	0.8	0.7 – 1.0	0.059
No	1.0		
Cats on Farm			
Yes	0.8	0.6 - 1.1	0.129
No	1.0		
Pigs on Farm			
Yes	1.1	0.8 - 1.6	0.548
No			
Breed			
Holstein	0.9	0.8 - 1.6	0.504
Other	1.0		
<i>Season</i>			
Summer	1.0	0.8 – 1.3	0.887
Winter	0.9	0.7 – 1.2	0.500
Spring	1.0		
Consistency of the feces			
Dry	1.1	0.2 - 5.2	0.923
Runny	0.9	0.2 – 4.5	0.883
Liquid	1.9	0.4 – 9.6	0.442
Blood	0.8	0.1 – 5.6	0.840
Normal and loose	1.0		

the shedding of *G. duodenalis* among animal in the study where shedding was at 24.4%, 24.7%, and 22.9% during Spring, Summer, and Winter, respectively (Table 1).

Results of univariate analysis of putative risk factors associated with *Giardia* shedding are shown in Table 1. From the distribution of age in Fig. 1 the age an animal was categorized into four categories that coincide with management practices (1–30 days; 31–60 days; 61–180 days; and greater than 180 days). There was a significant association between the age of the animal and the likelihood of shedding *G. duodenalis*. Calves below 30 days of age had five times the risk of shedding *G. duodenalis* in comparison to cattle above 180 days of age (OR = 4.6, 95% CI 3.4–6.1, $p < 0.0001$). Young stock (31–60 days of age) had the highest risk in comparison to adult animals (OR = 12.3, 95% CI 8.8–17.1, $p < 0.0001$). Younger heifers (61–180 days of age) were at increased risk in comparison to adult animals (OR = 4.3, 95% CI 3.3–5.8, $p < 0.0001$).

There was a significant association between concurrent infection with *Cryptosporidium* spp. and the likelihood of shedding *G. duodenalis* in the univariate analysis (Table 1). Animals co-infected with *G. duodenalis* and *Cryptosporidium* spp. were 1.8 times more likely to be infected with *G. duodenalis* compared to those that were not co-infected (OR = 1.81, 95% CI 1.26–2.60, $p < 0.0001$). Season of sampling and having other animals (cats or pigs) on the farm were not associated with the likelihood of shedding *Giardia* cysts (Table 1). We also examined the potential association between the consistency of the feces and the likelihood of shedding *G. duodenalis*. There was no significant association between the consistency of the feces and the likelihood of shedding *Giardia* cysts (Table 1).

We evaluated the significance of association between the likelihood of shedding *G. duodenalis* cyst and the putative factors that were found to be significant in the univariate analysis to assess the potential confounding effect of each factor while controlling for other

Table 2. Factors associated with the likelihood of shedding *G. duodenalis* while controlling for the presence of other factors.

Factor	Regression coefficient	Standard Error	Odds ratio	95% Confidence interval
Intercept	-1.872	0.11		
<i>Age</i>				
≤ 31 days	1.537	0.147	4.6	3.5 – 6.7
31 - 60 days	2.537	0.170	12.6	9.1 – 17.6
61 – 180 days	1.497	0.145	4.5	3.4 – 5.9
> 180 days	0.0		1.0	
<i>Dogs</i>				
Present	-0.338	0.116	0.7	0.6 – 0.9
Absent	0.0		1.0	

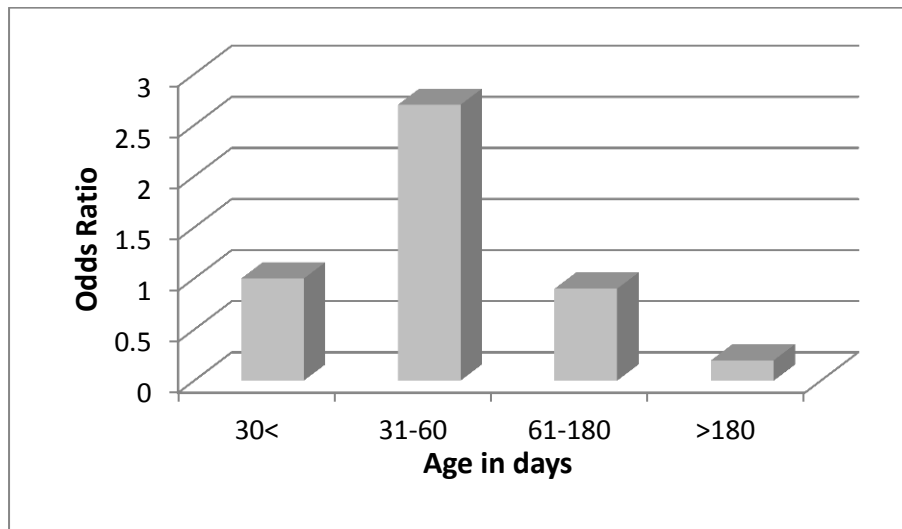


Figure 2. The relationship between the age of the animal and the probability of shedding *Giardia duodenalis* among cattle included in the study population

factors. The analysis was performed using logistic regression analysis and the results are shown in Table 2. Only the age of the animal and the presence of dogs on the premises were significantly associated with shedding. The likelihood of shedding increased with age, peaked at 30–60 days of age, and declined thereafter (Fig. 2). It was less likely to detect *G. duodenalis* in fecal samples from cattle on farms with dogs on the premises (Table 2).

The results of the analysis of the factors that affect the intensity of shedding *G. duodenalis* are shown in Table 3. There was no significant correlation among animals by farm in the intensity of shedding, meaning that the random effect parameter for farm was not significant. Three factors were found to be significantly associated with intensity of shedding: season of sampling, age of the animal, and co-infection with *Cryptosporidium* spp. The average shedding rate in the study population was 171 cysts per gram (95% CI 47–624). These values were computed from the antilog (base 10) of the intercept. Animals sampled in the spring shed significantly less cysts in comparison to those sampled in the winter (on average 3 cyst per gram). There was no significant difference in the amount of cysts shed per gram between animals sampled in summer and winter (Table 3). Animals 30 days of age or less and animals between 31 and 60 days of age shed more cysts per gram in comparison to animals that were older than 180 days (19 and 17 cyst per gram of feces, respectively). There was no significant difference in the number of cysts per gram shed by animals 61–180 days of age and animals older than 180 days (Table 3). Animals that were not co-infected with *Cryptosporidium* spp. shed 4 cysts per gram/feces on average in comparison to animals that were co-infected (Table 3).

A total of 304 of the 504 flotation-positive samples were analyzed by PCR. Bands corresponding to the *tpi* and β -*giardin* amplicons were visualized on agarose gels for 131 samples (43.1%) representing 23 of the 34 farms in the study. Genotyping identified 29 samples

(22.1%) as assemblage A and 102 samples as assemblage E (77.9%). Assemblage B was not found in any of the samples. Both assemblages A and E were identified on four farms, assemblage A only on one farm, and assemblage E only on 18 farms (Table 4). All of the samples that were identified as assemblage A were from calves under 84 days of age. We were able to subtype 26 of the samples identified as assemblage A based on BLAST analysis. Half were identified as AI and the other half as AII (data not shown).

4. Discussion

Giardia duodenalis was identified in fecal samples from cattle from all of the 34 farms that participated in the study. The overall prevalence was slightly lower than other cross-sectional studies (Castro-Hermida et al., 2006 and Santín et al., 2009), however, our study represents a repeated cross-sectional study (a version of a follow-up study), enrolled a larger sample size, employed a different sampling scheme, and used a different diagnostic test. The prevalence of *G. duodenalis* in dairy calves fell into the range of previously published reports that varied from 22% to 58% (Geurden et al., 2008, Hamnes et al., 2006, Santín et al., 2009 and O’Handley et al., 1999). Individual farm prevalence varied from 5% to 42% and seemed to be related to the number of calves sampled. The farm with the lowest overall prevalence of *G. duodenalis* did not have any calves on the farm at the time of sampling, whereas the farm with the highest overall prevalence had 92% of positive samples that came from calves.

Data on putative risk factors were obtained by personal interview of the owners/managers of the farm using a questionnaire. Information about the exact age of the animal was sometimes not available; however, we were able to obtain approximate ages consistent with the categories used in the analysis. Using this approach we were able to retain these samples in the statistical

Table 3. Linear regression analysis of factors that affect the intensity of *Giardia* cyst per gram shedding measured in the log base 10.

Factor	Estimated parameter	Standard Error	p-value	95% CI	Geometric mean shedding and 95% CI (cysts per gram feces)
Intercept	2.232	0.287	0.0001	1.669 – 2.795	171 (47, 624)
<i>Season</i>					
Spring	-0.411	0.168	0.015	-0.741 - -0.080	3 (1, 5) ^a
Summer	-0.183	0.168	0.277	-0.513 – 0.147	2 (1, 3) ^a
Winter	0.0				
<i>Age</i>					
≤ 31 days	1.271	0.199	0.001	0.881 – 1.661	19 (8, 46)
31 - 60 days	1.228	0.190	0.001	0.853 – 1.600	17 (8, 40)
61 – 180 days	0.296	0.192	0.124	-0.081 – 0.672	
> 180 days	0.0				
<i>Cryptosporidium</i> infection					
No	-0.608	0.239	0.011	-1.076 - -0.140	4 (1, 12) ^a
Yes	0.0				

Table 4. Assemblages of *G. duodenalis* found in cattle samples.

Sequence Accession	Gene	Farm Number (number of <i>Giardia</i> positive samples)	Assemblage
AB469365	<i>β-giardin</i>	8(1), 22(2), 25(2)	A
AY655702	<i>β-giardin</i>	20(4)	A
EU726988	<i>β-giardin</i>	20(1), 31(1)	A
AY368157	<i>tpi</i>	20(1)	A
EF654693	<i>tpi</i>	20(1)	A
FJ560560	<i>tpi</i>	22(1)	A
FJ560569	<i>tpi</i>	20(9)	A
AY655703	<i>β-giardin</i>	1(1), 2(1), 8(1), 23(1), 24(2), 25(2), 28(1), 31(1), 32(1), 34(3)	E
AY072729	<i>β-giardin</i>	1(1), 8(1), 18(1), 24(5), 27(1), 28(2), 31(1), 34(2)	E
DQ116615	<i>β-giardin</i>	15(1)	E
DQ116620	<i>β-giardin</i>	19(1), 27(1), 34(2)	E
DQ116621	<i>β-giardin</i>	1(1), 9(1), 12(1), 18(1), 19(1), 20(1), 24(5), 25(2), 26(3), 27(1), 28(3), 34(4)	E
DQ116623	<i>β-giardin</i>	18(1), 24(1), 26(1), 28(2), 34(1)	E
DQ116624.1	<i>β-giardin</i>	12(3), 17(1)	E
DQ116625	<i>β-giardin</i>	6(1), 25(2), 28(1), 34(1)	E
EU726984.1	<i>β-giardin</i>	24(1)	E
EU726986.1	<i>β-giardin</i>	12(1), 24(1), 26(1)	E
EU726987.1	<i>β-giardin</i>	20(1)	E
FJ472822	<i>β-giardin</i>	24(1)	E
GQ337972.1	<i>β-giardin</i>	32(1)	E
EF654684	<i>tpi</i>	4(1)	E
EF654689	<i>tpi</i>	4(1), 14(1)	E
EF654692	<i>tpi</i>	2(1), 4(1), 12(2), 22(1)	E
EU189326	<i>tpi</i>	22(1)	E
EU189375	<i>tpi</i>	20(1), 23(2), 24(1), 26(1), 28(1), 32(1), 34(1)	E
EU726982	<i>tpi</i>	9(1)	E
EU726984	<i>tpi</i>	9(1), 33(1)	E
EU781025	<i>tpi</i>	2(1)	E

analyses. The finding regarding the age association and the likelihood of shedding *G. duodenalis* cysts in this study is consistent with the findings in other studies (Hamnes et al., 2006, Winkworth et al., 2008a, Winkworth et al., 2008b and Mark-Carew et al., 2010). Season was not shown to be significantly associated with infection. The year was divided into three seasons by grouping months with similar climate and weather patterns. Grouping the months according to standard definitions of seasons may have led to different results.

In the multivariate analysis, the presence of dogs on the farm and age were the only two factors that were associated with the likelihood of shedding *G. duodenalis*. The latter finding is consistent with the pattern reported in the literature by others (Wade et al., 2000; Mark-Carew et al., 2010, Coklin et al., 2010). The negative association between the presence of dogs on the farm and the likelihood of shedding begs for speculative explanation. The obvious explanation is that this variable would be a proxy to another factor that we did not control for in this study.

The PCR efficiency for this study was 43.1% (131/304). If a sample did not amplify the gene fragment of interest well on the first attempt, the PCR reaction was run again (if no band was seen) or re-amplified from the secondary reaction (if a faint band was seen after the secondary reaction). After two failed attempts, the sample was considered not amplifiable. Many factors may have played a role in failed PCR amplification. Samples were collected during different seasons, transported from the farms to the laboratory for different periods of time, were processed at different times and with different lots of DNA extraction kits. It is quite possible that some samples degraded over time. PCR inhibitors found in fecal samples may have also been a cause for lack of amplification.

Initially, the *tpi* gene was used for molecular characterization of *Giardia* DNA. There was some difficulty amplifying the gene so β -*giardin* PCR primers were used for subsequent

samples. The transition resulted in more robust DNA amplification, indicated by stronger bands on the gel. Occasionally, multiple bands would appear other than the one corresponding to the size of *β-giardin* amplicons. In such cases, the Qiagen Gel Extraction Kit was used to purify DNA from the correct band prior to sequencing.

All samples containing assemblage A of *G. duodenalis* were from calves under 84 days of age highlighting the possible role of calves in the potential zoonotic transmission of the parasite. Based on available literature, this is the first report in which no adult cattle were infected with assemblage A. Since some of the positive samples were not PCR-amplified or sequenced, the prevalence of both assemblage A and E in this study cannot be fully reported. The actual prevalence of assemblage A may be underestimated since only 22 of the 34 farms were represented by sequenced samples.

Age information was obtained for 100 of the sequenced samples of which 97 were from calves under 6 months of age. 95.8% of samples identified as assemblage E were from calves 6 month of age or less. Calves in this study population were separated from their mothers shortly after birth. It is possible that calves obtained infections from their mothers, yet the fact that no adult cows in this study were infected with assemblage A leads us to believe that younger animals shedding the zoonotic strain of *G. duodenalis* are acquiring infections from other sources, possibly other animals on the farm, wildlife or humans. Because *Giardia* is a waterborne disease it is possible for infections of the zoonotic strains (assemblages A and B) to spread within the barn due to the animals sharing areas designated for water. An interesting addition to our study would be to collect fecal samples from other animal species to determine if similar (sub)assemblages of *G. duodenalis* are found among all of the animal populations on the farms.

Interpreting chromatogram results proved difficult when there was overlap of two peaks or the absence of a peak. Effort was made to avoid the problem of incorrect nucleotide selection by analyzing both DNA strands. In cases where multiple peaks were present at the same position on both strands, the nucleotide with the highest peak on the chromatogram was selected. Multiple peaks may signify mixed infections, allelic sequence heterogeneity, or genetic recombination (Sprong et al., 2009). This study did not take into account the possibility of mixed infections in dairy animals. It is possible that assemblage A went undetected in some positive samples. Assemblage A-specific primers are available for the *tpi* gene (Levecke et al., 2008) and could have been used to identify the zoonotic genotype in fecal samples.

The PCR primers we used to amplify the *tpi* and β -giardin amplicons were able to identify different assemblages of *G. duodenalis* and subtypes of assemblages A. The AI subtype is commonly found in a variety of mammalian species including cattle (Leonhard et al., 2007, Volotão et al., 2007 and Langkjaer et al., 2007). However, there are a handful of published reports of the AII subtype in cattle (Mendonça et al., 2007 and Feng et al., 2008). Overall, AI and AII differ significantly in their host preference with AII infections predominantly found in humans (Sahagún et al., 2008 and Xiao and Fayer, 2008). There is still debate about the anthroponotic and zoonotic potential of assemblage A infections in dairy cattle. Future directions point to more comprehensive molecular characterization of *G. duodenalis* assemblages at the individual nucleotide level.

Cattle co-infected with *Cryptosporidium* spp. (*C. parvum* and *C. andersoni*) and *G. duodenalis* were almost 2 times more likely to be infected with *G. duodenalis* based on univariate analysis. However, when taking other factors into consideration, co-infection was no longer statistically significant. We previously reported in an incidence study that dairy cattle

from herds with prior infections with both protozoa were more likely to become infected with *G. duodenalis* when compared to herds with no prior history of *Giardia* or *Cryptosporidium* infection and herds with only *Giardia* infections (Mark-Carew et al., 2010). We believe that *Giardia* and *Cryptosporidium* are linked epidemiologically based on their mode of transmission, clinical manifestations, and their potential for zoonotic infections. The statistical significance of co-infection in this study helps to support this theory.

Prior work assessing infections of dairy cattle with *G. duodenalis* in the New York City Watershed has been done, but this study represents the first attempt to characterize *G. duodenalis* assemblages in dairy herds in this region. The results show that potentially zoonotic strains of *G. duodenalis* are prevalent in dairy herds, particularly in young animals, and that further investigation into the epidemiology of *Giardia* infections is needed.

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CHAPTER FOUR *

MULTI-LOCUS CHARACTERIZATION OF *GIARDIA DUODENALIS* ASSEMBLAGES IN DAIRY CATTLE IN TRINIDAD AND TOBAGO: THE POTENTIAL AGRICULTURAL RISK OF *GIARDIA* INFECTIONS TO HUMANS

* In the format of Zoonoses and Public Health

Summary

- *Giardia duodenalis* is one of the most common gastrointestinal parasites of humans, wildlife, companion animals and livestock, namely, dairy cattle.
- While research on the potential zoonotic risk of *G. duodenalis* from dairy cattle has been conducted worldwide, there are no known reports from Trinidad and Tobago. The goals of this work were to determine the prevalence of *G. duodenalis* in dairy cattle in the country, assess the potential zoonotic risk of this protozoan to humans by characterizing assemblages of *G. duodenalis* in the study population through multi-locus analyses, and to identify risk factors that may perpetuate infections in dairy animals.
- Because cattle can harbor zoonotic strains of *G. duodenalis* and excrete high numbers of cysts into the environment, it is important to identify and characterize strains of *G. duodenalis* in dairy animals in Trinidad and Tobago so that the potential risk of *Giardia* infections to humans from cattle can be mitigated.

Abstract

Giardia duodenalis infections in dairy cattle have been reported worldwide, however, the presence of infections in these animals in Trinidad and Tobago is unknown. There is increasing evidence of the zoonotic potential of the parasite and the possibility that cattle are reservoirs for human infections. In this cross-sectional study, we aimed to assess the potential zoonotic risk associated with *Giardia* isolates recovered from cattle on both islands of Trinidad and Tobago and identify risk factors that may be associated with infections. A total of 273 fecal samples were collected from dairy animals on 20 farms of which 196 samples were analyzed by PCR amplification of the *ssu-rRNA* gene. The overall prevalence of *G. duodenalis* by PCR was 12.8% (25/196) with 14 farms (70%) having at least one animal shedding cysts at the time of sampling. All positive samples were subjected to additional PCR analysis at the beta-giardin (*bg*) locus for multi-locus characterization of *G. duodenalis* infections. Ten samples had PCR products of the correct size. There was assemblage agreement between the *ssu-rRNA* and *bg* loci for seven isolates, with three isolates being characterized as assemblage A at the *ssu-rRNA* locus and as assemblage E at the *bg* locus. This discrepancy may indicate mixed infections in these samples. Assemblages A and E were identified while assemblage B was not detected in the study population. Of significance was that two samples had genotypes of *G. duodenalis* not previously deposited in Genbank. Age and co-infection with at least one other parasite were significantly associated with being *Giardia* PCR-positive from univariate analysis ($p < 0.05$). Given the presence of assemblage A in dairy animals from our study population, the zoonotic risk of *G. duodenalis* infections from dairy animals to humans needs to be further investigated in Trinidad and Tobago.

Introduction

Giardia is a common intestinal parasite of animals. Of the six species of *Giardia* presently recognized in the literature (*G. agilis* in amphibians, *G. ardeae* and *G. psittacci* in birds, *G. microti* in muskrats and voles, *G. muris* in rodents, and *G. duodenalis* in various mammalian species), only one, *Giardia duodenalis* is infectious to humans (Adam, 2001; Monis et al., 2009). High prevalence of infection with *G. duodenalis* is often reported in domesticated mammals such as companion animals and livestock. The parasite is routinely identified as the main source of parasitic load in ruminants, particularly, dairy cattle (Feng and Xiao, 2011). Additionally, *G. duodenalis* is a frequently implicated pathogen of waterborne outbreaks worldwide. Considering the possible connection between outbreaks of human disease and the presence of farm operations near water sources, recent studies have addressed the potential of cattle to serve as reservoirs for human infections (O’Handley et al., 2006; Trout et al., 2007).

The zoonotic potential of *G. duodenalis* has been the subject of much debate as host specificity of the organism remains unclear (Erlandsen et al., 1988; Thompson, 2000; Plutzer et al., 2010). Many studies suggest that there is a risk of zoonotic transmission of *Giardia* from infected cattle to humans (Learmonth et al., 2003; Khan et al., 2011; Cardona et al., 2011, Budu-Amoako et al., 2012). Isolates of *Giardia* recovered from dairy cattle feces are morphologically and antigenically identical to human isolates, making it difficult to distinguish between livestock-specific genotypes and those that infect humans by microscopy (Buret et al., 1990).

Advancements in molecular biology have allowed for genetic characterization of *Giardia* spp. from different host species (Cacciò et al., 2008a). Recent phylogenetic studies have subdivided *G. duodenalis* into eight distinct assemblages, five of which have tight host specificity and three with broad host ranges. Assemblages A and B infect a wide-array of

mammals including wildlife, companion animals, livestock, and humans with more species diversity for Assemblage A infections (Feng and Xiao, 2011). Assemblages C and D infect wild and domestic canids, assemblage E infects domestic ruminants and pigs, assemblage F infects cats, assemblage G infects rodents, and assemblage H infects seals (Cacciò et al., 2005; Lasek-Nesselquist, et al., 2010).

Because assemblages A and B are considered zoonotic strains, analysis of genetic differences within each assemblage may provide insight on the relationship between subtypes and hosts, the zoonotic potential of each subtype, contamination source tracking, and transmission dynamics of *G. duodenalis* (Feng and Xiao, 2011). Based on polymerase chain reaction (PCR) sequence analyses of three genes—the triose phosphate isomerase (*tpi*), the glutamate dehydrogenase (*gdh*), and the beta-giardin (*bg*) genes—multiple subtypes of assemblage A were determined. The division of assemblage A into AI, AII and AIII is supported by phylogenetic analyses done at the *gdh* locus, and within each subgroup are subassemblage-specific polymorphisms. The subgroups of assemblage A appear to differ in host affinity. While different mammalian species have been infected with both AI and AII, humans are more commonly infected with AII (Cacciò et al., 2008b). AIII has predominantly been identified in wildlife and hoofed animals with no known human infections (van Giessen et al., 2006; Cacciò et al., 2008b).

In contrast to assemblage A, there is currently no clear subgrouping of assemblage B. Subassemblages BIII and BIV were described using allozyme electrophoretic studies, not by DNA sequence analyses (Feng and Xiao, 2011). Dairy cattle infections with assemblage B are much less-frequent in the literature and have been documented in Europe, China, Canada, and

New Zealand (Learmonth et al., 2003; Lalle et al., 2005; Coklin et al., 2007; Winkworth et al., 2008).

Prevalence data on *G. duodenalis* infections in dairy cattle have been documented worldwide. Heterogeneity of infection rates is evident within and between countries. In studies done in the United States, Canada, and New Zealand, infection rates have ranged from 19.1% to 52%, 8.7% to 57%, and 4.5% to 40.6%, respectively (O’Handley et al., 2000; Trout et al., 2005; Coklin et al., 2007; Winkworth et al., 2008b; Santín et al., 2009). Factors such as study design, age of animals, location, method of diagnosis, and management practices contribute to the reported variability of prevalence rates (Wade et al., 2000; Maddox-Hyttel et al., 2006; Miller et al., 2007; Mark-Carew et al., 2012). Longitudinal studies that followed young dairy animals over a specified period of time reported high incidence rates within study herds (Trout et al., 2009; Mark-Carew et al., 2010; Paz e Silva et al., 2012). Cross-sectional studies have also indicated high infection rates, especially within-farm prevalence rates (Trout et al., 2005). *G. duodenalis* is highly prevalent in dairy calves with infection rates as high as 100% in some herds (Xiao and Herd, 1994; O’Handley et al., 1999). Prevalence of assemblages of *G. duodenalis* also varies from study to study. Reports of *G. duodenalis* infections in dairy cattle show infections with assemblage A, E, A and E, or mixed infections of the two assemblages within individual animals, with most studies showing concomitant infections of A and E within the study population (Trout et al., 2004; Trout et al., 2005; Feng et al., 2008; Geurden et al., 2008; Mark-Carew et al., 2011). Subassemblage information is less available in the literature with limited reports of AI and AII in dairy animals (Feng and Xiao, 2001; Khan et al., 2011).

While *G. duodenalis* infections have been studied in several countries and in a variety of species, to our knowledge, reports from the Caribbean are absent in the literature. The risk of

infections to humans from agricultural animals has not been studied in the region. Here, we present the first report of *Giardia* infections in dairy cattle in Trinidad and Tobago. The present study sought to 1) determine the prevalence of *G. duodenalis* across dairy operations on both islands of the country, 2) identify the zoonotic potential of these isolates recovered from dairy animals using a multi-locus genotyping approach, 3) and to examine putative risk factors that may be associated with infections.

Material and Methods

Study Design and Sample Collection

A cross-sectional study was conducted to address the stated objectives. The target population was dairy herds on both islands of Trinidad and Tobago. The sampling frame consisted of 20 farms of which 14 were private farms and 6 were government farms (research or extension). Eighteen farms were located in Trinidad and two were in Tobago. Since no prior prevalence studies on *G. duodenalis* in dairy cattle in Trinidad and Tobago could be found in the literature, the sample size was determined to be 139 animals with an estimated prevalence of 10% of cows shedding *G. duodenalis*. A proportional age-stratified sampling scheme was adopted whereby twenty dairy animals were selected from each farm for sampling based on age; 10 were collected from calves (animals younger than six months of age) and 10 from heifers and adults (animals greater than six months of age). If less than 20 animals were on the farm, we attempted to collect as many samples as were available.

Samples were collected per rectum from 273 dairy animals from October 2010 to June 2011 using a convenience sampling method. At the time of collection, samples were collected in plastic zip-lock bags labeled with the animal's identification number. They were stored on ice

during transport from the farm to the University of the West Indies School of Veterinary Medicine where they were stored at 4°C prior to processing.

Sample analyses

All samples were analyzed using a qualitative centrifugation concentration flotation technique with zinc sulfate (1.18 sg) and sucrose (1.33 sg) as the flotation media. Briefly, 5-10 grams of feces were added to 40 mL of water and the solution was mixed into a slurry. Tea strainers were used to remove debris and the resulting liquid was added to two 15 mL conical tubes. Tubes were centrifuged and the supernatants were removed. The pellet was disrupted with the addition of either zinc sulfate or sucrose filling each tube to a slightly positive meniscus. Coverslips were placed on tubes and left standing on the benchtop for 10 minutes. Visual examination was done using bright-field microscopy. This technique facilitated diagnoses of larger parasites such as coccidia and helminths. Slides were rinsed with water and washings were poured into 1.5 mL centrifuge tubes for storage and subsequent processing.

DNA Extraction

DNA was extracted from 196 samples using the Qiagen Blood and Tissue Kit (Valencia, CA) following a slightly modified protocol. 180 µL of ATL Buffer and 20 µL of Proteinase K were added to 1.5 mL tubes containing 50 µL of diluted feces from slide washings. Following an overnight incubation at 56°C, the manufacturer's protocol was followed. The only exception was that 100 µL of nucleic acid was eluted using AE buffer instead of the suggested 200 µL. DNA extracted samples were stored at -20°C.

Sample Transport

Diluted fecal samples and DNA extractions were transported from the University of the West Indies School of Veterinary Medicine (Mt. Hope, Trinidad and Tobago) to Cornell

University (Ithaca, NY, USA) on dry ice by commercial air transport. At Cornell University, they were stored at -20°C until further analyses.

PCR and DNA sequencing

All DNA-extracted samples were analyzed using polymerase chain reaction (PCR) of the *ssu-rRNA* locus as the primary method of diagnosis. A 292 or 150 base-pair fragment of the *ssu-rRNA* gene was amplified using a previously described nested PCR protocol (Hopkins et al., 1997; Read et al., 2002). Positive samples were subjected to additional PCR analyses to compare PCR efficiency and genetic characterization at different loci. A 511 base-pair fragment from the *bg* loci was amplified using a nested PCR protocol (Lalle et al., 2005). All reactions contained 5 µl of DNA. A high cyst count *Giardia* positive sample from a previous study was used as a positive control while a reaction mixture containing all essential elements for the PCR minus DNA served as a negative control. PCR products were analyzed on 1% agarose gel with ethidium bromide staining. All positive PCR products were purified using Exonuclease I/Shrimp Alkaline Phosphatase (Exo-SAP-IT) (USB Corporation, Cleveland, OH) and sequenced in both directions in 18 µL reactions at the Cornell University Life Sciences Core Laboratories Center using the Applied Biosystems Automated 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). Sequence chromatograms of each strand were aligned and examined with MEGA-4 software (2008). Nucleotide sequences obtained in the present study were aligned with those in Genbank using BLAST (<http://www.ncbi.nlm.nih.gov/blast>) to compare *Giardia*-positive samples to those previously deposited in Genbank. New sequences representative of the study population were deposited in Genbank under accession numbers JQ781662 and JQ781663.

Statistical Analyses

Results from PCR amplification of the *ssu-rRNA* gene were used to determine the prevalence of *Giardia*-positive samples in the study population. The prevalence was computed as the proportion of samples that tested positive by PCR out of all the samples that were examined. The significance of association between potential risk factors and the likelihood of cattle shedding *G. duodenalis* in the feces was evaluated using univariate logistic regression. The magnitude of the association was quantified using the odds ratios (ORs) and interval estimates of the ORs were computed using the 95% confidence interval. Using the “PROC GLIMMIX” statement, a nested mixed model was created to account for the random effect of farms (SAS Statistical Software, Raleigh, NC). Categorical variables included age of animal (<6 months or ≥ 6 months), gender (male or female), season of sampling (dry or rainy), farm type (private or government), farm size (≤ 50 dairy animals or >50 dairy animals), location, and co-infection with other intestinal parasites (presence or absence of at least one other parasite). The significance level for the factors in all models was assessed at $p < 0.05$.

Results

A total of 25 samples out of 196 samples were diagnosed as shedding *Giardia* cysts based on PCR amplification of the *ssu-rRNA* gene (12.8%). Fourteen farms had at least one positive sample (70%). Within-farm prevalences ranged from 5.3% to 66.7% (Table 1). Of the 25 PCR-positive samples, 16 were identified as assemblage E and 9 as assemblage A. Two isolates had new genetic sequences that were deposited into Genbank at the *ssu-rRNA* locus. One new isolate was most homologous to HQ616609, and the other had most sequence homology to AY655701 (Figure 1). Accession numbers for sequences already deposited in Genbank identified in this study are JF957619 (assemblage A), AB469363 (Assemblage A), JF957620 (assemblage E), EU189371 (Assemblage E), FJ472822 (Assemblage E), and GQ329671 (Assemblage E).

Amplification of the *bg* locus resulted in lower PCR efficacy compared to amplification of the *ssu-rRNA* locus. Ten of the twenty-five samples (already sequenced and identified as *G. duodenalis*) had bands corresponding to the correct-sized band of the *bg* PCR amplicon (40%). BLAST results showed assemblage agreement in seven out of ten samples (Table 4).

Table 1. Within-farm prevalence and assemblages of *G. duodenalis* identified on 14 farms in the study. Age of infection animals is also included (Young = <6 months, Adult \geq 6 months).

Farm	Within-farm Prevalence (%)	<i>G. duodenalis</i> assemblages (subassemblage)	Age of animals
1	15.0	E	Young
2	33.3	A(I) and E	Young and Adult
3	10.0	E	Adult
4	12.5	E	Young
5	11.1	E	Adult
10	66.7	E	Young
12	5.6	E	Young
13	10.0	E	Young
14	5.3	A(I)	Young
15	33.3	A	Adult
16	16.7	E	Adult
18	16.7	A(I)	Young
19	9.1	A(I)	Adult
20	60.0	A and E	Adult

Table 2. Prevalence of parasites other than *G. duodenalis* found in all samples collected from dairy cattle based on microscopy (N=273).

Parasite	Number of Animals Infected	Prevalence (%)
Strongyles	81	29.7
<i>Eimeria</i> sp.	73	26.7
<i>Monezia</i> spp.	4	1.5
<i>Capillaria bovis</i>	2	0.07
<i>Toxocara vitulorum</i>	1	0.04

Table 4. Prevalence of *G. duodenalis* infections for each potential risk factor with corresponding odds ratios (OR) and 95% confidence intervals.

Risk factor	Total	Frequency	Prevalence (%)	OR	95% CI
Age					
<i>≤ 6 months</i>	57	11	19.3	2.32	0.097 – 5.538
<i>>6 months</i>	139	13	9.3	1.0	
Location					
<i>Trinidad</i>	181	23	12.7	2.04	0.255 – 16.24
<i>Tobago</i>	14	1	7.1	1.0	
Location					
<i>Carlsen Field</i>	47	6	12.8	0.838	0.271 – 2.383
<i>Wallerfield</i>	74	11	14.9	0.589	0.206 – 1.591
<i>Other</i>	75	7	9.3	1.0	
Gender					
<i>Male</i>	17	4	23.5	2.45	0.727 – 8.230
<i>Female</i>	179	20	11.1	1.0	
Season					
<i>Rainy</i>	89	13	14.6	1.49	0.633 – 3.512
<i>Dry</i>	107	11	10.3	1.0	
Farm Type					
<i>Private</i>	146	18	12.3	1.03	0.384 – 2.762
<i>Government</i>	50	6	12.0	1.0	
Farm size					
<i><50 animals</i>	107	13	12.1	1.49	0.633 – 3.519
<i>≥ 50 animals</i>	89	11	12.3	1.0	
Co-infection					
<i>Yes</i>	92	17	18.5	3.14	1.239 – 7.964
<i>No</i>	104	7	6.7	1.0	

Table 4. Results of multi-locus genotyping of samples at the *ssu-rRNA* and *bg* loci.

Isolate	<i>ssu-rRNA</i>		<i>bg</i>	
	Accession	Assemblage	Accession	Assemblage
G-SBC	JF957619	A	GQ329671	A
ME-11	JF957619	A	EU189371	E
STO-5/6	AB469363	A	EU189371	E
A-K19	JF957620	E	EU189371	E
A-K25	JF957620	E	FJ472822	E
B-OBC	JF957620	E	FJ472822	E
ME-15	JF957620	E	EU189371	E
U-9	JF957620	E	EU189371	E
O-3420	JF957620	E	EU189371	E

Within-farm prevalence and assemblages of *G. duodenalis* identified on 14 farms in the study are shown in Table 1. Categorical age of infected animals is also included (Young = <6 months, Adult \geq 6 months).

Based on microscopy, 28.2% of all samples collected had at least one other parasite (not including *Giardia*) present in fecal samples (77/273). Intestinal parasites included strongyle eggs, *Eimeria* spp., *Toxocara vitulorum*, *Capillaria bovis*, and *Monezia* sp. Strongyles were the most commonly detected parasites with 75 animals (27.4%) shedding eggs in their feces followed by *Eimeria* spp. with 70 (25.6%) animals shedding oocysts. Table 2 shows the prevalence of other parasites found in the study population.

Table 4 shows the prevalence and odds ratios of being PCR positive for *G. duodenalis* according to each risk factor analyzed prior to accounting for the effect of farm. Univariate analysis of each risk factor showed that cattle that were co-infected with *G. duodenalis* and at least one other intestinal parasite were significantly more likely to shed than animals that were not co-infected (OR = 3.14, 95% CI 1.24 – 7.96). Male cattle (OR 2.45, 95% CI 0.73 – 8.23) and younger cattle (OR = 2.32, 95% CI 0.097 – 5.538) were also more likely to be PCR-positive for *G. duodenalis*, however, the confidence intervals included “1” and were, therefore, not significant. Location, farm size, and farm type were not significantly associated with infection.

Univariate analyses in which the effect of farm was taken into account in the model resulted in age and co-infection being statistically significant. Gender was not considered significant because the p-value was just higher than our assigned α ($p = 0.0595$) (data not shown). In multivariate analyses, only co-infection remained significant, indicating a correlation between age and co-infection.

Discussion

G. duodenalis infections have worldwide distribution in both humans and animals. Given the various possible routes of transmission—waterborne, foodborne, anthroponotically and zoonotically—it is important to identify sources of infections and characterize infections at the molecular level and across geographical settings to obtain information about the assemblages of *G. duodenalis* found around the world. Additionally, because the zoonotic potential of the organism is constantly being reviewed, studies aimed at identifying and characterizing infections in different mammalian species will assist in understanding the risk of *G. duodenalis* to humans from other animals.

This study provides preliminary data on *G. duodenalis* infections in dairy cattle in Trinidad and Tobago and the potential of dairy cattle to serve as reservoirs for zoonotic infections in humans. The overall prevalence for the study was 12.8%. We sampled over the required sample size (N=139) by collecting 273 samples and analyzing 196 of them. Our study prevalence is on the low end of the range of infection rates reported in dairy cattle worldwide (Trout et al., 2005; Geurden et al., 2008; Winkworth et al., 2008). One recent report from Brazil showed a prevalence of 7.5% with a similar number of samples analyzed and the same method of diagnosis (Paz e Silva et al., 2012).

The low prevalence of *G. duodenalis* infections detected in the study may be attributed to several factors. Firstly, we did not use microscopy for visual identification of *Giardia* cysts. It is possible that animals shedding low numbers of cysts went undiagnosed by PCR of the *ssu-rRNA* gene, our sole method of diagnosis. To account for this, we used what we regarded as a high volume of DNA, 5 µl, for each PCR reaction. Additionally, we believe that simply using PCR as our reference diagnostic test allowed for high sensitivity and specificity. Secondly, our study

JQ781663	CATCCGGTCGATCCTGCCGGAGCGGACGCTCTCCCCAAGGACGAAGCCATGCATGCCCG	60
AY655701	CATCCGGTCGATCCTGCCGGAGCGGACGCTCTCCCCAAGGACGAAGCCATGCATGCCCG	60
JQ781662	CATCCGGTCGATCCTGCCGGAGTGCAGCTCTCCCCAAGGACGAAGCCATGCATGCCCG	60
HQ616609	CATCCGGTCGATCCTGCCGGAGCGGACGCTCTCCCCAAGGACGAAGCCATGCATGCCCG	60
	*****↑*****	
JQ781663	CTACCCGGGACGCGGGACGGCTCAGGACGACGGTTGCACCCCGCGGGCGGTCCCTG	120
AY655701	CTACCCGGGACGCGGGACGGCTCAGGACGACGGTTGCACCCCGCGGGCGGTCCCTG	120
JQ781662	CTACCCGGGACGCGGGACGGCTCAGGACAAAGGTTGCACCCCGCGGGCGGTCCCTG	120
HQ616609	CTACCCGGGACGCGGGACGGCTCAGGACAAAGGTTGCACCCCGCGGGCGGTCCCTG	120
	*****↑*****	
JQ781663	CTAGCCGGACACCGCTGGCAACCCGGGCGCAAGACGTGCGCGCAAGGGCGGGCGCCGCG	180
AY655701	CTAGCCGGACACCGCTGGCAACCCGGGCGCAAGACGTGCGCGCAAGGGCGGGCGCCGCG	180
JQ781662	CTAGCCGGACACCGCTGGCAACCCGGGCGCAAGACGTGCGCGCAAGGGCGGGCGCCGCG	180
HQ616609	CTAGCCGGACACCGCTGGCAACCCGGGCGCAAGACGTGCGCGCAAGGGCGGGCGCCGCG	180

JQ781663	GGCGAGCAGCGTGACGACGACGGCCCGCCGGGCTTCCGGGGCATCACCCGGTCGGCG	240
AY655701	GGCGAGCAGCGTGACGACGACGGCCCGCCGGGCTTCCGGGGCATCACCCGGTCGGCG	240
JQ781662	GGCGAGCAGCGTGACGACGACGGCCCGCCGGGCTTCCGGGGCATCACCCGGTCGGCG	240
HQ616609	GGCGAGCAGCGTGACGACGACGGCCCGCCGGGCTTCCGGGGCATCACCCGGTCGGCG	240

JQ781663	CGGTCCGCGGCGCGCCGAGTGCCCGACGCTGGCGGAGAA	279
AY655701	CGGTCCGCGGCGCGCCGAGTGCCCGACGCTGGCGGAGAA	279
JQ781662	CGGTCCGCGGCGCGCCGAGTGCCCGACGCTGGCGGAGAA	279
HQ616609	CGGTCCGCGGCGCGCCGAGTGCCCGACGCTGGCGGAGAA	279
	*****↑*****	

Figure 1. ClustalX alignment of new isolate sequences (JQ781663 and JQ781662) compared to previously published sequences (AY655701 and HQ616606) identified as having the greatest homology from BLAST analyses. Arrows indicate sites of discrepancies between the four sequences.

design was such that we attempted to include an equal number of calves under six months of age and animals six months and older. Yet, only 57 animals were designated as calves for statistical analyses. Having a study population with mostly calves would have likely resulted in a higher prevalence since younger animals are more likely to shed *Giardia* cysts.

Because no prior prevalence data existed for *G. duodenalis* infections in dairy cattle in Trinidad and Tobago, we assumed a prevalence of 10%, an underestimation based on our results. The inclusion of more farms in the study, and, therefore, more animals, would have provided us with more power in our ability to detect *G. duodenalis* infections. Finally, we used a convenience sampling method in this study. On some farms, less than twenty animals were available for sampling, with some farms having no calves. Additionally, some animals did not provide feces at the time of sampling. With all of these factors considered, it is possible that our reported prevalence is an underestimation of the true prevalence of *G. duodenalis* in dairy cattle in Trinidad and Tobago.

Age and co-infection with another intestinal parasite were the only two factors analyzed that were significantly associated with the likelihood of shedding *G. duodenalis*. We have previously reported an association between co-infection of *G. duodenalis* and *Cryptosporidium* spp. (Mark-Carew et al., 2010). However, we did not test for the presence of *Cryptosporidium* spp. in this study. That study was done on dairy animals in the United States. Further studies are needed to determine whether *Giardia* and *Cryptosporidium* infections in dairy cattle in Trinidad and Tobago are epidemiologically linked.

Season was not significantly associated with infection (OR = 1.49, 95% CI, 0.633 – 3.512). Some published reports have shown associations between weather and infection (Wade et al., 2000; Jagai et al., 2010; Mark-Carew et al., 2012). Season was divided based on

meteorological information on the duration of the rainy and dry seasons in Trinidad and Tobago. Given that the temperature in Trinidad and Tobago remains fairly consistent throughout the year, we speculated that the differing accumulation of rainfall between the wet and dry seasons would have led to a strong association between the wet season and *G. duodenalis* infections. The type of farm and size of farm did not appear to play a role in infections in dairy cattle. A major limitation to our study was that information on management factors were not collected for statistical analyses. Certain practices such as hand-milking, spreading of manure, and calf isolation may have been associated with infections in the study population.

Assemblages A and E were found in the study population with most dairy animals being the livestock-specific assemblage E. Assemblage B was not identified in the study population, lending to further evidence of absence of this assemblage in most of the Western Hemisphere with the exception of one study on dairy cattle in Canada (Coklin et al., 2007). We identified novel strains of *G. duodenalis* from two samples based on amplification and sequencing of the *ssu-rRNA* locus, each representing a single base-pair difference from already published sequences (Figure 1). Unfortunately, PCR amplification of these samples at the *bg* locus was unsuccessful after two attempts, preventing multilocus analyses.

We were able to compare assemblage results based on BLAST analyses of deposited sequences in Genbank for ten samples (Table 3). For seven samples, identification of either assemblage A or E matched at both the *ssu-rRNA* locus and the *bg* locus. For isolates M-11, STO-5, and STO-6, BLAST analyses matched with 100% homology to sequences identified as Assemblage A at the *ssu-rRNA* locus and with 100% homology to sequences identified as assemblage E at the *bg* locus. It is possible that these samples consisted of mixed infections of

assemblages A and E of *G. duodenalis* since both assemblages were found in other dairy animals on these farms (Table 1).

All isolates identified as assemblage A in the study fell into subassemblage AI based on published sequences at the *ssu-rRNA* locus. AI classification was not confirmed at the *bg* locus based on BLAST analyses. Additional analyses using PCR-restriction fragment length polymorphism (RFLP) would have provided data on subassemblages at this locus, as has been done in other studies (Lalle et al., 2005; Khan et al., 2011). We did not attempt to classify subtypes of assemblage E found in the study because we were mostly interested in highlighting the zoonotic potential of *G. duodenalis*.

With regard to the risk of human infections from dairy cattle, we believe there is concern for zoonotic spread of *G. duodenalis* infections. Calves have been shown to begin shedding *Giardia* cysts as early as 2 days of age and can shed as high as 10^6 cysts per gram feces, potentially making them long-term, high-output reservoirs for infection (O’Handley et al., 2003; Mark-Carew et al., 2010). Adult cattle should not be excluded as sources of infections since only 10 cysts are needed to propagate infections and the majority of *Giardia*-positive animals in this study were adults (Rendtorff, 1954; Schaefer et al., 1991). In this study, both young and adult dairy animals shed assemblage A (Table 3). Of the ten animals that shed assemblage A, three were over 6 months of age (age for one sample was unknown). While calves are more likely to be infected, older cattle may help to propagate infections within these herds.

Based on our sampling strategy, for some herds, only a sub-population of animals was sampled. Therefore, it was not possible to evaluate the endemicity of *G. duodenalis* infections at the farm level. We cannot rule out the possibility that animals that were not sampled were infected. Additionally, given the cross-sectional nature of the study, we may have missed

infections at other points during the life of the dairy animals in the study. Longitudinal studies that track dairy animals in Trinidad and Tobago over a period of time may shed further light on the dynamic of infections in this population.

This is considered the first report of *G. duodenalis* in dairy cattle from Trinidad and Tobago. The presence of assemblage A infections in the study population indicate a potential risk of *Giardia* infections in humans from dairy cattle. Further studies are needed in humans and other livestock in the country to characterize the agricultural risk of *G. duodenalis* to humans at a molecular level.

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CHAPTER FIVE *

POTENTIAL ZOONOTIC RISK OF *GIARDIA DUODENALIS* FROM COMPANION ANIMALS: THE RISK OF INFECTION FROM DOGS IN TRINIDAD AND TOBAGO

* In the format of Veterinary Parasitology

Abstract

To our knowledge, the zoonotic potential of *Giardia duodenalis* has not been assessed in companion animals in Trinidad and Tobago. This report details the first attempt to evaluate the potential zoonotic risk of *G. duodenalis* from dogs and identify assemblages of *G. duodenalis* found in dog populations on both islands. Fecal samples were collected from free-roaming dogs and dogs at the Trinidad and Tobago Society for the Prevention of Cruelty to Animals from October 2010 to June 2011. Descriptive information, such as age, gender, season of collection, and ownership status (owned, shelter, free-roaming, relinquished), were recorded at the time of collection to determine risk factors associated with infection. 168 samples were collected of which 104 samples were analyzed for the presence of *G. duodenalis* by polymerase chain reaction (PCR) with subsequent assemblage-typing. A total of 26 samples were positive for *G. duodenalis* based on PCR amplification of the *ssu-rRNA* gene for an overall prevalence of 25%. Four samples were identified as assemblage C (15.4%), 21 as assemblage D (80.8%), and one as assemblage E (3.8%). As expected, puppies were significantly more likely to be infected with *G. duodenalis* than adult dogs (OR 4.61, 95% CI 1.73 – 12.2). We infer from our results that while the prevalence of *G. duodenalis* is relatively high in Trinidad and Tobago, the zoonotic risk of infection in humans is negligible since neither assemblage A nor B was identified in the study population.

Introduction

Giardia is a widespread parasite associated with gastrointestinal disease of humans, wildlife, livestock, and companion animals. It is estimated that 200 million people worldwide, particularly, in Latin America, Africa, and Asia, are infected with *Giardia* each year. In developed countries, giardiasis is considered an emerging infectious disease due to its association with gastrointestinal disease from several water- and foodborne outbreaks (WHO, 1996). Symptoms include dehydration, diarrhea, abdominal pain, nausea, and weight loss (Robertson et al., 2010).

The genus *Giardia* includes several species that infect numerous hosts, including mammals, amphibians and birds. The taxonomy of *Giardia* has been subject to review over the years, with over 50 different species at one time described in the literature. Currently, six species have been accepted by researchers: *G. agilis* (amphibians), *G. ardeae* (birds), *G. muris* (rodents), *G. microti* (muskrats and voles), *G. psittacci* (birds), and *G. duodenalis* (wide host range of mammalian species). *Giardia duodenalis* is further divided into assemblages. Assemblages A and B are considered the potentially zoonotic strains since they infect several mammalian species including humans, wildlife, livestock and companion animals. Assemblage C and D infect domestic and wild canids, assemblage E infects hooved livestock, assemblage F infects cats, assemblage G infects rodents, and assemblage H infects seals (Feng and Xiao, 2011).

The zoonotic potential of *G. duodenalis* has been investigated in various mammalian species, particularly, dogs. While host-specific assemblages C and D are often cited, a growing number of reports show assemblages A and B in dog populations around the world (Paoletti et al., 2008; Cooper et al., 2010; Upjohn et al., 2010; Volotão et al., 2011; Berrilli et al., 2012). In a survey of 296 *G. duodenalis* infections in dogs in the United States, 28% were assemblage A and

41% were assemblage B (Covacin et al., 2011). A study done in Germany showed assemblage A was the most prevalent strain found in dogs (Leonhard et al., 2007). A handful of community-based studies have shown assemblage A and B infection in humans and dogs living in the same locality (Hopkins et al, 1997; Traub et al., 2004; Impankaew et al., 2007; Traub et al., 2009; Cooper et al., 2010). Furthermore, a study done in Thailand, revealed that 79% of dogs and 73% of humans tested were infected with assemblage A, implicating dogs as reservoirs for human *G. duodenalis* infections.

In canine populations, *G. duodenalis* is often the most common enteric parasite, yet it is not usually associated with clinical disease (Palmer et al., 2008). Overall infection rates in dogs differ based on many variables, including the composition of dog populations (owned/free-roaming/kennel) and the type and sensitivity of the test used for diagnosis. Reports of infections range from 0.1% in owned dogs to as high as 100% in kenneled dogs. Puppies, free-roaming dogs, and shelter dogs have a higher risk for infection than adult dogs and owned dogs (Stehr-Green et al., 1987; Dubná et al., 2007; Mircean et al., 2012).

Various diagnostic tests have been used to identify *Giardia* infections in dogs. Zinc sulfate centrifugation with subsequent microscopy is most commonly used for detection and visualization of *Giardia* cysts. Immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA) are used to detect *Giardia* surface antigens. A growing number of reports in the literature describe the use of ELISA for diagnosing infections (Rishniw et al., 2010; Upjohn et al., 2010; Olson et al., 2010; Itoh et al., 2011). PCR, however, is the most sensitive diagnostic tool, allowing for molecular characterization of *Giardia* assemblages and subassemblages. In a study where researchers determined prevalence rates based on microscopy

and PCR, microscopy yielded a prevalence of 3% while PCR resulted in 20% of samples being diagnosed as *Giardia*-positive (Traub et al., 2004).

The present study was conducted to identify *Giardia duodenalis* infections in dogs in Trinidad and Tobago. The aims were to determine the prevalence among owned, shelter, and free-roaming dog populations, to identify risk factors associated with *Giardia* infections, to characterize strains of *G. duodenalis* present in dogs on both islands of the country, to evaluate the agreement between ELISA and PCR in the diagnosis of this protozoan, and to determine the prevalence of other parasites in the study population.

Materials and Methods

Study design and sample collection

A cross-sectional study was carried out to address the stated objectives. The target population consisted of dogs in Trinidad and Tobago. The study population included free-roaming dogs as well as dogs at both branches of the Trinidad and Tobago Society for the Prevention of Cruelty to Animals (TTSPCA) in St. James, Trinidad and Lowlands, Tobago. Prior to the start of the study, the sample size was determined to be 139 animals given an estimated 10% prevalence of *G. duodenalis* infection in dogs in the target population. Since no prior cross-sectional studies had been conducted to study *Giardia* infections in dogs in Trinidad and Tobago, the prevalence estimate was based on published literature from other countries (Eligio-García et al., 2008; Katagiri et al., 2008; Lebbad et al., 2008; Gingrich et al., 2010; Klimbel et al., 2010; Soriano et al., 2010).

Any dog at the TTSPCA with a fecal specimen in the kennel at the time of the collection was included in the study. Demographic information about each dog was available on kennel doors. For free-roaming dogs only information on gender, location of collection, approximate

age (puppy or adult), and type of housing was available. Fecal samples were collected from 168 animals using a convenience sampling method over an eight-month period from October 2010 to June 2011. At the time of collection, samples were placed in zip-lock bags, labeled with the name of each dog, and stored on ice in a cooler. They were then stored short-term (1-2 days) at 4°C prior to microscopy.

Sample analysis and Microscopy

All samples were processed using a qualitative centrifugation concentration flotation technique with zinc sulfate (1.18 sg) and sucrose (1.30 sg) as the flotation media. Microscopic examination was conducted using bright-field microscopy. This technique facilitated diagnoses of larger parasites such as helminth eggs and coccidia oocysts. Slides were rinsed with water and the washings were poured into 1.5 mL centrifuge tubes for storage and subsequent processing.

DNA extraction

DNA was extracted from 104 samples using the Qiagen Blood and Tissue Kit (Valencia, CA) following a slightly modified protocol. 180 µL of ATL Buffer and 20 µL of Proteinase K were added to 1.5 mL tubes containing 50 µL of diluted feces from slide washings. Following an overnight incubation at 56°C, the manufacturer's protocol was followed. The only exception was that 100 µL of nucleic acid was eluted using AE buffer instead of the suggested 200 µL. DNA extracted samples were stored at -20°C.

PCR and DNA sequencing

All DNA-extracted samples were analyzed using PCR as the primary method of diagnosis. A 150 bp fragment of the *ssurRNA* gene was amplified using a previously described nested PCR protocol (Hopkins et al., 1997; Read et al., 2002). PCR products were analyzed on 1% agarose gel with ethidium bromide staining. All positive PCR products were purified using

Exonuclease I/Shrimp Alkaline Phosphatase (Exo-SAP-IT) (USB Corporation, Cleveland, OH) and sequenced in both directions in 18 µL reactions at the Cornell University Life Sciences Core Laboratories Center using the Applied Biosystems Automated 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). Sequence chromatograms of each strand were aligned and examined with MEGA-4 software (2008). Nucleotide sequence searches were conducted using BLAST (<http://www.ncbi.nlm.nih.gov/blast>) to compare *Giardia*-positive samples with those previously deposited in Genbank.

ELISA

The Remel ProSpect *Giardia* Microplate Assay was used for antigenic detection of *Giardia*. ELISAs were conducted on diluted fecal samples that were *Giardia* positive from PCR with subsequent sequencing. This was done to evaluate the sensitivity of ELISA given that the samples were subjected to conditions not recommended by the manufacturer's protocol. Samples were thawed, and cotton swabs were used to mix the solution. Swabs were added to specimen dilution buffer, and the manufacturer's protocol was followed thereafter. Both visual observation and plate reader results were used to determine *Giardia* positive samples. 22 PCR positive samples and 14 PCR negative samples were tested.

Statistical Analyses

Results from PCR amplification of the *ssu-rRNA* gene were used to determine the prevalence of *Giardia*-positive samples from the study population. The prevalence was computed as the proportion of samples that tested positive by PCR out of all the samples that were examined. The significance of association between each potential risk factor and the likelihood of dogs shedding *G. duodenalis* in the feces was evaluated using univariate logistic regression. The magnitude of the association was quantified using the odds ratios (ORs) and

interval estimates of the OR were computed using the 95% confidence interval. Backward stepwise logistic regression analysis was performed to assess the significance of association between the likelihood of shedding *G. duodenalis* and each of the factors that were found significant in the univariate analysis while accounting for the presence of the other factors. Factors that were analyzed included age (puppy or adult), gender (male or female), location (Trinidad or Tobago), ownership status (free-roaming, shelter, or owned/relinquished), season of sample collection (rainy season or dry season), assemblage type (C or D), and co-infection with at least one other parasite (based on microscopy). Data were analyzed using JMP Pro 9.0.2 (SAS Statistical Software, 2010). The significance level for the factors in all models was assessed at $p < 0.05$.

Diagnostic agreement between PCR and ELISA was determined using the Kappa statistic (κ). The test measured the percent agreement between the two tests that occurred beyond chance. A κ value less than .40 indicates poor agreement between test, between .40 and .75 indicate fair to good agreement, and greater than .75 indicates excellent agreement. The κ statistic is:

$$\kappa = \frac{2(ad - bc)}{p_1q_2 + p_2q_1}$$

where a represents the proportion of samples in which both test resulted in a positive diagnosis, b represents the proportion of samples in which PCR resulted in a positive diagnosis and ELISA was negative, c represents the proportion of samples in which ELISA resulted in a positive diagnosis and PCR was negative, d represents the proportion of test in which both PCR and ELISA resulted in negative diagnoses. p_1 represents the proportion of samples that were PCR-positive, q_1 represents the proportion of samples that were PCR-negative, p_2 represents the proportion of samples that were ELISA-positive, and q_2 represents the proportion of samples that were ELISA-negative (Fless, 1981; Sim and Wright, 2005). Assuming that the sensitivity and

specificity of PCR were both 100%, the sensitivity, specificity, positive predictive value, and negative predictive value were determined for ELISA.

Results

A total of 26 samples were identified to be positive for *Giardia* by PCR amplification of the *ssu-rRNA* gene out of 104 total samples tested for an overall prevalence of 25%. Table 1 shows the breakdown of prevalence by ownership status, age, gender, and season of sampling. DNA sequencing revealed three assemblage types of *G. duodenalis* found in dog fecal samples. 4 samples were identified as assemblage C (15.4%), 21 as assemblage D (80.8%), and one as assemblage E (3.8%). Zoonotic assemblages were not found in the study population. All assemblage C *Giardia* positive samples were from puppies, while assemblage D came from both puppies and adult dogs. The Genbank accession numbers were AB569372 (assemblage C), AB569371 and DQ890189 (assemblage D), and HQ283229 (assemblage E).

Based on microscopy, 50.6% of all samples collected (85/168) had at least one parasite, excluding *G. duodenalis*. Fecal parasites included eggs of hookworms, *Toxocara canis*, *Trichuris vulpis*, *Dipylidium caninum*, *Diocotophyma renale*, *Cystisopora canis*, and *Spirocerca lupi*. Adult *Demodex* mites were also detected. 65.4% of *Giardia* PCR-positive samples had at least one other parasite detected in the feces. *Ancylostoma caninum* was the most commonly detected parasite. The prevalence of helminth infections in PCR-analyzed samples ranged from 1.0% to as high as 34.6% (Figure 1).

Univariate analyses of each risk factor showed that puppies were significantly more likely to shed *Giardia* than adults (OR 4.61, 95% CI 1.73 – 12.2, $p = 0.0018$) (Table 1). Adult dogs and puppies from Trinidad were significantly more likely to shed *Giardia* in comparison to

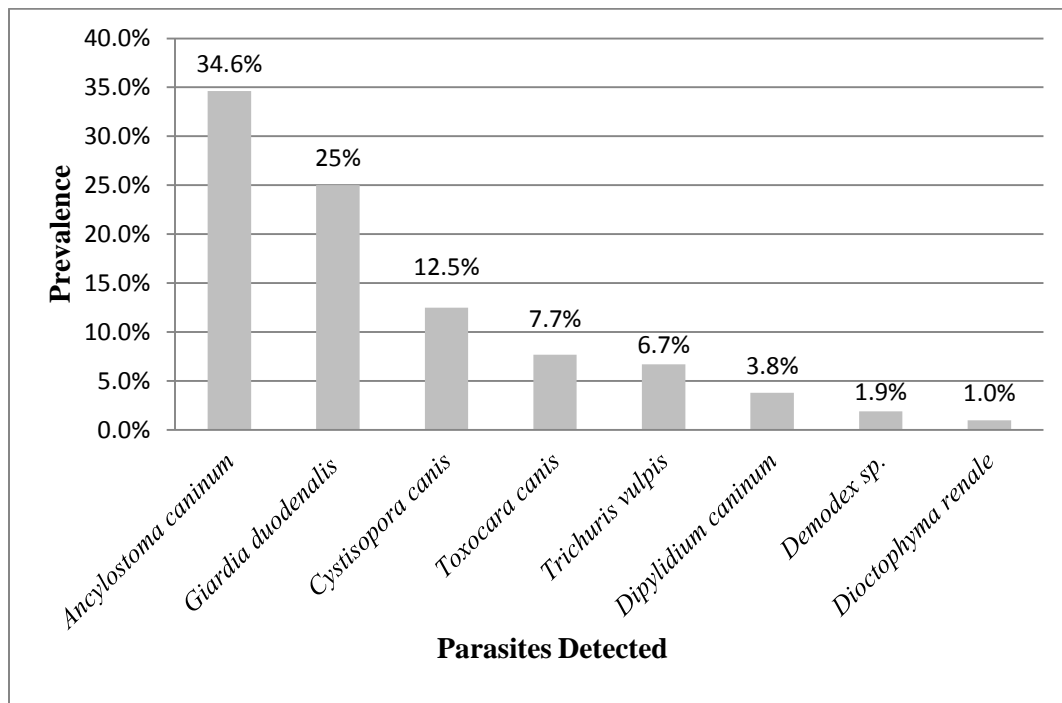


Figure 1. Prevalence of parasites in fecal samples that were analyzed by PCR (n=104). PCR was used to determine the prevalence of *G. duodenalis* while microscopy was used to determine all other parasite infection rates.

Table 1. Prevalence and odd ratios of *Giardia* positive samples according to each risk factor.

Risk factor	Total	Frequency	Prevalence	OR	95% CI
Age					
<i>Puppy</i>	31	14	45.2%	4.61	1.73 – 12.2
<i>Adult</i>	66	10	15.5%	1.0	
Location					
<i>Trinidad</i>	89	25	28.1%	5.47	0.68 – 43.8
<i>Tobago</i>	15	1	6.7%	1.0	
Housing					
Individual	96	21	21.9%	5.95	1.31 – 26.97
Group	8	5	63%	1.0	
Gender					
<i>Male</i>	48	12	25%	1.41	0.53 – 3.83
<i>Female</i>	46	9	19.6%	1.0	
Season					
<i>Dry</i>	38	12	31.6%	1.71	0.68 – 4.23
<i>Rainy</i>	66	14	21.2%	1.0	
Status					
<i>Free-roaming</i>	11	6	54.6%	8.4	1.80 – 45.20
<i>Shelter</i>	42	12	28.6%	2.8	0.86 – 10.95
<i>Owned/Relinquished</i>	32	4	12.6%	1.0	
Co-infection					
<i>Yes</i>	49	14	28.6%	1.43	0.59 – 3.49
<i>No</i>	55	12	21.8%	1.0	

Table 2. Multiple logistic regression using backward stepwise elimination.

Factor	Estimate	Standard Error	p-value	Adjusted Odds Ratio	Adjusted 95% CI
Intercept	0.496	0.533			
Age					
Adult	0				
Puppy	0.666	0.320	0.0349	3.8	1.1 – 14.2
Status					
Shelter or owned	0				
Free-roaming	1.086	0.378	0.0035	8.8	2.05 – 42.0
Housing					
Individual	0				
Group	1.064	0.478	0.0192	8.4	1.41 – 69.8

Table 3. The kappa statistic for the test agreement between PCR and ELISA.

		ELISA		
		+	-	
PCR	+	16	6	0.611
	-	0	14	0.389
		0.444	0.556	
Kappa		Standard Error	95% CI	p-value
0.674699		0.114639	0.45 – 0.90	<0.0001

dogs from Tobago (OR 5.47, 95% CI 0.68 – 43.8, $p = 0.0477$) (Table 1). The results of multivariate analysis are shown in Table 2. The risk of infection with *G. duodenalis* was influenced by the age, ownership status, and system of management. Puppies were twice more likely to be infected with *G. duodenalis* in comparison to adult dogs when we accounted for the source of the dog and the system of management. Free-roaming dogs are almost nine times more likely to be infected with *G. duodenalis* in comparison to shelter or privately own dogs (OR =8.8) when we accounted for the age of the animal and method of housing. Group housing was associated with increased likelihood of infection with *G. duodenalis* in comparison to individual dog housing when we accounted for the risk associated with the age and ownership status of the dog.

A total of 16 of the 22 samples that were *Giardia*-positive by PCR and sequencing were positive by ELISA; 15 of 22 were positive from the microplate reader with one “suspect” (scored as positive from visualization). Diagnostic agreement between PCR and ELISA (using visualization) was 0.67, indicating fair to good agreement between the two tests (Table 3). The sensitivity of ELISA was 72.7%, specificity was 100%, positive predictive value was 100% and negative predictive value was 70% given the method of fecal sample processing used in this study.

Discussion

This is the first report of assemblages of *G. duodenalis* infections in dogs in Trinidad and Tobago. The 25% prevalence obtained from the samples collected from free-roaming dogs and dogs at the TTSPCA was much higher than we anticipated with our sample size calculations. Given that there was no prior literature on *Giardia* infections in dogs in Trinidad and Tobago, we were limited in our ability to accurately determine the sample size and used 10% as the estimated

prevalence based on previously published prevalence data on the presence of *Giardia* in owned, shelter, and free-roaming dogs from several countries.

Most *Giardia*-positive samples were sequenced as Assemblage D. For this study, phylogenetic analyses were not conducted since no zoonotic strains were identified in the study population. Also, we were not able to account for mixed infection with the PCR primers used in this study. Future prevalence studies on *Giardia* infections in dogs in Trinidad and Tobago should utilize sequence analyses of various loci of *G. duodenalis* DNA, such as the beta-giardin (*bg*), triosephosphate isomerase (*tpi*), and glutamate dehydrogenase (*gdh*) genes, to not only determine assemblages, but subassemblages of assemblages A, B, C, and D. We found it interesting that only puppies were infected with assemblage C. Adult dogs were 8.14 times more likely to shed assemblage D of *G. duodenalis* when we corrected for a “0” cell in the contingency table (adult dogs with Assemblage C) using the Woolf-Haldane correction (data not shown). To our knowledge, no one has reported an association between age and *Giardia* assemblage types in dogs.

Some descriptive studies have shown how social and environmental conditions may contribute to assemblage types of *G. duodenalis* shed by dogs, in particular, their interactions with humans (Hopkins et al., 1997; Leonhard et al., 2007). However, these studies were designed to present preliminary molecular information about the types of *G. duodenalis* assemblages found in dogs without attempting to make epidemiological conclusions through statistical analyses. Our study is one of a few studies in the literature to make associations between specific risk factors and assemblage types of *G. duodenalis* present in dogs.

Although assemblage E has been reported in domestic cats (Read et al., 2004; Sprong et al., 2009; Lebbad et al., 2010), to our knowledge, it has never been found in dogs. The one

sample from our study population sequenced as assemblage E came from a dog described only as a male Shepherd mix with a fecal sample collected during the dry season. We can only speculate that this dog became infected by some interaction with livestock.

In the current study, *G. duodenalis* infections were shown to be significantly higher in puppies than in adult dogs, a finding supported by published reports (Hamnes et al., 2007; Scaramozzino et al., 2009; Mircean et al., 2011; Itoh et al., 2011). Furthermore, it has been established that the higher infection rates in younger animals is common across mammalian species (Bartlett et al., 1991; Spain et al., 2001; Hamnes et al., 2007; Mark-Carew et al., 2010).

For some of our hypothetical risk factors, the number of samples may have been too low to draw conclusions about *G. duodenalis* in the target population, all dogs in Trinidad and Tobago. For example, only 15 samples were collected from Tobago. Missing information resulted in many samples being excluded from univariate analyses. Additionally, information such as fecal consistency and vaccination and deworming records, which may have been associated with *G. duodenalis* infections based on our regression models, were not collected.

ELISA was able to detect 16 positive samples of 22 positive samples tested. Fresh fecal samples are recommended for analysis by the manufacturer's protocol. The samples in this study underwent several levels of processing, were very dilute, and were frozen and thawed more than once prior to testing. We acknowledge that the sensitivity and negative predictive value of the ELISA would have likely been higher under appropriate testing conditions.

The zoonotic potential of *G. duodenalis* from dogs in the study population may be negligible since neither assemblage A or B were found. It is hypothesized that dog-specific assemblages may outcompete zoonotic strains in kennels, pet shops, and shelters, whereas zoonotic strains may predominate in dogs that often interact with humans and human

environments (Ballweber, 2010). Several studies have shown this to be the case (Hopkins et al., 1997; Lalle et al., 2005; Leonhard et al., 2007; Eligio-García et al., 2008; Silva et al., 2012). The majority of the study samples were dogs classified as shelter dogs, 12 of which were shedding dog-specific *Giardia* strains. Follow-up studies that include dogs with close interactions with human populations may shed some light on the cross-transmission of *G. duodenalis* among these populations.

Our study revealed a high prevalence of *G. duodenalis* in dogs from Trinidad and Tobago. Although dog-specific assemblages predominated in the study population, a more comprehensive study with a larger sample size and more predictor variables may contribute to better understanding of the ecology and zoonotic potential of *G. duodenalis* infections in dogs in the country.

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CHAPTER SIX
CONCLUSIONS

Systematic epidemiologic approaches are essential to addressing and rectifying public health concerns, especially with regard to zoonoses. While the zoonotic potential of *G. duodenalis* continues to be controversial, the findings presented in this dissertation contribute to growing knowledge about the ecology and epidemiology of *G. duodenalis* in animal populations. Infection rates of *G. duodenalis* above 10% were reported for all four studies conducted. Dairy herds in the New York City Watershed (NYCW) had high incidence rates of 25% and 23.9% for the two cohort studies. For the cross-sectional studies done in Trinidad and Tobago, the prevalence of *G. duodenalis* infection was 12.8% and 25% in dairy cattle and dogs, respectively. There should be a general concern about the transmission of *G. duodenalis* from these populations to humans given these high rates. Genetic characterization of *G. duodenalis* found in infected animals, as done in three of the four studies of this dissertation work, is currently the best approach to determining the risk of zoonotic infections of *G. duodenalis*.

The first study provided baseline data on *Giardia* infections in dairy cattle in the NYCW, representing one of the largest *Giardia* incidence studies in dairy cattle to date. Over 10,600 samples were collected from 4,938 dairy animals. A major finding was the association of *Cryptosporidium* infections with the likelihood of shedding *Giardia*, suggesting that these protozoa may be epidemiologically linked. Cattle from the high-risk cohort (identified as cattle from farms previously having both *Cryptosporidium* and *Giardia* infections) had the lowest probability of remaining *Giardia*-free over the course of the study, were significantly associated with shedding *Giardia*, and were significantly associated with high intensity of cyst-shedding. Additional risk factors included age and season of shedding.

The second study was a follow-up to the first study and included the target population of dairy cattle in the NYCW. The major aim was to genetically characterize *Giardia* infections

found in the watershed to determine if potentially infectious strains exist in the water that is supplied to inhabitants of New York City. Over the course of the study, 2,109 samples were collected from dairy animals on 34 farms. PCR was utilized on 304 samples of which 131 had amplicons of the correct size at the *tpi* or *bg* locus. 22% of samples were identified as assemblage A and 78% as assemblage E. A major finding was that all cattle harboring assemblage A were calves under the age of 84 days, demonstrating that calves may be reservoirs for potentially zoonotic strains of *G. duodenalis*.

The third study addressed the prevalence of *G. duodenalis* infections in dairy herds in Trinidad and Tobago and identified *G. duodenalis* infections in this study population through multilocus analyses of the *ssu-rRNA* and *bg* loci. Of 196 samples tested using PCR, 25 were positive for *G. duodenalis* at the *ssu-rRNA* locus. Of the 25 samples, 10 amplified at the *bg* locus. Assemblage homology at both loci was evident for seven out of ten samples upon DNA sequencing. Three samples were identified as assemblage A at the *ssu-rRNA* locus and as assemblage E at the *bg* locus, suggesting that these animals had mixed infections. Two new strains of *Giardia* were deposited to Genbank (Accessions JQ781662 and JQ781663), adding new genetic information about the global distribution of assemblage A and assemblage E infections.

The fourth study was designed to address the risk of *Giardia* infections to humans from various dog populations in Trinidad and Tobago. Samples were collected from free-roaming dogs and local animal shelters on both islands comprising owned (boarded), shelter, and free-roaming dogs in the country. The prevalence of *G. duodenalis* was 25% with 26 of 104 samples identified as *Giardia*-positive by PCR amplification of the *ssu-rRNA* gene. Four samples were identified as assemblage C, twenty-one as assemblage D, and one as assemblage E. This was the

first report of assemblage E in a dog based on a thorough search of published literature. Additionally, all of the samples identified as assemblage C were from puppies, suggesting a preference of this assemblage for younger dogs. Risk factors shown to be significantly associated with *Giardia* infections included age, group housing, and ownership status (free-roaming). Confidence intervals of estimates in this study were wide, indicating that a larger sample size would have resulted in improved precision and greater power to detect infections. Finally, comparison of PCR and ELISA resulted in a kappa statistic of 0.67, corresponding to good test agreement. It is important to note that the ELISAs were run on very dilute samples and that the ability of the test to accurately diagnose infections (Sensitivity = 70%, Specificity = 100%) is noteworthy.

This dissertation adds to the body of knowledge about *Giardia* infections Western Hemisphere. The implementation of molecular diagnostic tools in diagnosing and characterizing infections allowed for greater understanding of the epidemiology of *G. duodenalis* infections and the public health risk associated with infections of animal origin. Further molecular epidemiologic studies, cross-sectional and longitudinal, are needed to conclude whether or not *G. duodenalis* shed from animals poses a risk to humans.